LOCATION OF ANTIGENIC DETERMINANTS AND EXPRESSION OF THE ENVELOPE GLYCOPROTEIN OF MURRAY VALLEY ENCEPHALITIS VIRUS

by

Eva Lee

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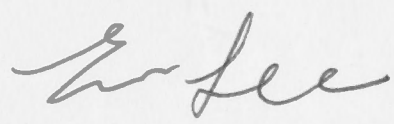
Statement

All the work reported in this thesis was performed by myself in the Department of Biochemistry, the Faculty of Science, Australian National University, except for the selection of neutralization escape variants and the virulence studies presented in Chapter 4. The selection of variants was performed by Dr. R. C. Weir and Dr. J. T. Roehrig, and the virulence studies were performed jointly with Dr. I. D. Marshall at the John Curtin School of Medical Research.



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Abstract

A major aim of the work described in this thesis is to study functional determinants in the Murray Valley encephalitis virus envelope proteins. As a first step in developing an expression system for Murray Valley encephalitis virus proteins, randomly generated MVE cDNA fragments were expressed in *E.coli* (Chapter 2). By subcloning a partial Sau3A-digest of a cDNA clone coding for the MVE proteins C, prM, E, NS1, NS2A, NS2B and NS3 (Dalgarno *et al.*, 1986) into the pEX expression plasmids (Stanley and Luzio, 1984), recombinant plasmids which expressed ß-galactosidase-MVE fusion proteins were generated. Fusion proteins were screened with hyperimmune ascitic fluid against MVE and identified by sequence analysis of recombinant plasmids. Fusion proteins which contained prM-E, E-NS1-NS2A , E and NS2A were characterized. With the exception of an unstable clone containing the largest insert (prM-E; 2.8 kb), fusion proteins were expressed in yields of 5-40 mg per litre of induced culture. Little evidence for degradation of fusion proteins was observed.

The neutralization epitopes in MVE envelope protein (E) were examined using the above expression system by two methods (Chapter 3). Fusion proteins containing various fragments of E were generated by deletion mutation and the reactivity of five neutralizing mAbs (defining E-1c, E-1d, E-5b, E-7 and E-8 epitopes; Hawkes *et al.*, 1988; J. T. Roehrig, personal communication) with these fusion proteins determined. These five mAbs reacted specifically with unreduced MVE E protein in Western blots. However they differed in reactivity with reduced E. Only anti-E-8 mAb reacted with reduced MVE; the reactivity of other mAbs with MVE was lost. This approach allowed the mapping of epitope E-8 to a region between Tyr 201 and Pro 224 in E fusion proteins. The four other mAbs did not react with the E fusion proteins.

The second method was based on immunization experiments in mice. By injecting mice with partially purified fusion proteins containing E fragments Asp 22-Val 272,

Tyr 201-Pro 224 and Leu 327-Gly 429, antisera which reacted with fusion proteins and with MVE by ELISA were generated indicating that the MVE polypeptides present in these fusion proteins were immunogenic. However, no significant neutralization activity was detectable in these antisera indicating that the fusion proteins were unable to elicit neutralizing antibodies. We conclude that the E fusion proteins generated using the pEX vectors could be used to map particular epitopes only and to generate protein-specific antisera. However they were unsuitable for studies on functional activities of E as they did not assume the native conformation of E.

In order to define neutralization epitopes E-1c, E-1d, E-5b and E-7, sequence analysis of mAb-selected escape variants was used (Chapter 4). Neutralization escape variants of MVE (selected by Dr. R. C. Weir with the anti-E-1c mAb) were sequenced in the M and E genes. Single amino acid changes at Phe 274, Ser 276 and Ser 277 were found. Previous studies mapped Ala 126 as part of the E-1c epitope (S. H. Hartley and R. C. Weir, personal communication). We conclude that the E-1c epitope is composed of residues far apart on the primary sequence of E. The epitopes E-1c and E-8 mapped for MVE are in a domain which corresponds to domain A of TBE (Mandl *et al.*, 1989b). Variants resistant to anti-E-1d, E-5b and E-7 were selected (by Dr. J. T. Roehrig) using a different method to that used in the above study. These each contained multiple changes in E including the common changes Asp 134→Asn and Ala 358→Thr as well as additional changes His 263→Tyr (for E-1d variants) and Thr 51→Ala or Asn 197→Asp (for E-5b variants). Although one E-7 variant contained Thr 310→Ser change, the other variants were only changed at residues 134 and 358. We could not determine the changes responsible for resistance to neutralization by anti-E-1d, E-5b and E-7 mAbs.

In view of the possible overlap between neutralization epitopes and determinants of mouse virulence, seven sequenced antigenic variants (above) were tested for virulence in

21-day old mice inoculated by the intracerebral (ic) and intraperitoneal (ip) routes. The resulting ic/ip LD50 ratios indicated that two of seven variants were probably of reduced virulence. The ic/ip LD50 ratios for these two variants, P4a1 and P5a1 which contained single amino acid changes Ser 276→Arg and Ser 277→Ile respectively, were approximately 10- and 1000-fold less than that for the parent, MVE-1-51. Thus residues 276 and 277 may define a virulence determinant in MVE E protein.

The development of a eukaryotic expression system for the MVE structural proteins was explored in Chapter 5. A recombinant baculovirus containing the MVE structural protein genes downstream from the polyhedrin promoter was generated. Infection of *Spodoptera frugiperda* (Sf9) cells with this recombinant virus produced MVE proteins which corresponded to the envelope proteins prM and E seen in MVE-infected cells. Pulse-labelling experiments demonstrated efficient cleavage of polyprotein precursors to E. Using tunicamycin, it was demonstrated that recombinant E underwent Asn-linked glycosylation and that inhibition of glycosylation affected the cleavage of precursors into E in Sf9 cells. Recombinant E reacted with all five neutralizing mAbs directed against MVE E-1c, E-1d, E-5b, E-7 and E-8 epitopes. Reactivities were comparable on a molar basis to reactivities against E in MVE-infected cells. We conclude that the baculovirus expression system generated MVE E protein in a native conformation. The yield of E was 4-5 μg per 10^6 Sf9 cells. Recombinant E in Sf9 cells was distributed in the perinuclear region and on the cell surface.

Abbreviations

C6/36	mosquito *Aedes albopictus* cell line
CBA	competitive binding assay
cDNA	complementary DNA
C-terminal	Carboxy-terminal
DEAE	diethylaminoethyl
ddNTP	dideoxynucleoside triphosphate
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbant assay
EMEM	Eagle's minimal essential medium
FCS	foetal calf serum
FIA	Freund's incomplete adjuvant
HBSS	Hank's balanced salt solution
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid
HI	haemagglutination-inhibition
ic	intracerebral
ip	intraperitoneal
K	kilodaltons
kb	kilobases
LAH	lactalbumin hydrolysate
LB medium	Luria-Bertani medium
LD50	50% lethal dose
LMT	low melting temperature
mAb	monoclonal antibody
ß-ME	ß-mercaptoethanol
min	minutes

MOI	multiplicity of infection
mol. wt.	molecular weight
mRNA	messenger RNA
N-terminal	amino-terminal
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol
PFU	plaque forming unit/s
p.i.	post infection
poly-A	polyadenylic acid
PRNT	plaque reduction neutralization test
RE	restriction enzyme
RER	rough endoplasmic reticulum
RIP	radio-immunoprecipitation
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SW13	human adenocarcinoma cell line
Tris	Tris (hydroxymethyl) aminomethane
Vero	African green monkey kidney

Abbreviations of flaviviruses are given in Table 1.1.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Flaviviruses and their medical significance

The Flaviviridae is a recently established family of small enveloped RNA viruses (Westaway *et al.*, 1986). On the basis of structural features, serological reactions and replication strategy, at least 68 viruses have been classified as flaviviruses (Westaway *et al.*, 1986). Of the presently recognized flaviviruses, 28 cause diseases in humans and eight are associated with diseases in domesticated and wild animals of economic importance (see review by Monath, 1986). The most prominent examples are dengue fever, yellow fever and Japanese encephalitis which are diseases in humans caused by mosquito-transmitted flaviviruses (dengue virus, DEN; yellow fever virus, YF; and Japanese encephalitis virus, JE). Flavivirus disease symptoms in humans can vary from a mild febrile illness to the more severe encephalitis and hemorrhagic fever.

In Australia, the most prominent flavivirus disease is caused by Murray Valley encephalitis virus (MVE), which has been responsible for epidemic outbreaks of encephalitis in human with a focus of activity in the Murray Valley region in South-eastern Australia. Important outbreaks occurred in 1951 and 1974 during which 19 and 12 fatal human cases were reported, respectively (Marshall, 1988).

Flaviviruses of veterinary importance include JE, Wesselsbron virus and Louping ill virus. In animals, encephalitis in horses, stillbirth and abortion in pigs are caused by JE in many parts of Asia, while Wesselsbron virus and louping ill virus are associated with disease in lambs and sheep, respectively (Monath, 1986).

Prevention of flavivirus diseases depends largely on vaccination of susceptible human or animal populations, control of arthropod vectors responsible for transmission and protection against mosquito bite or tick bite. As flaviviruses are able to replicate and cycle in several host species, eradication of flavivirus diseases which pose serious public health problems is difficult. Currently, effective vaccines are only available for YF, tick-borne encephalitis (TBE) and JE; the YF vaccine is an attenuated strain of YF

(17D; Theiler and Smith, 1937), TBE and JE vaccines are derived from inactivated viruses (Kunz *et al.*, 1980; Okuda *et al.*, 1975). There is also an attenuated live JE vaccine in use in China (Yu *et al.*, 1988).

1.2 Relationships between flaviviruses

1.2.1 Serology

Flaviviruses can be grouped by their natural hosts (mosquitoes, ticks, bats and rodents), geographical distribution (Africa, Europe, Asia, Australia and North America), disease symptoms in human and animals and serological cross-reactions (Calisher *et al.*, 1989). The commonly accepted scheme for subdivision of flaviviruses is based on antigenic differences determined primarily by cross-neutralization tests with polyclonal antisera (De Madrid and Porterfield, 1974; Calisher *et al.*, 1989). The current scheme comprises eight antigenic complexes. These are the TBE complex (12 viruses), the Rio Bravo complex (6), the JE complex (10), the Tyuleniy complex (3), the Ntaya complex (5), the Uganda S complex (4), the DEN complex (4) and the Modoc complex (5). There are 17 other viruses apparently not closely related to any of the eight complexes (see Table 1.1). The YF is one of these. Members of the same antigenic complex generally show cross-neutralization with polyclonal antisera; cross-neutralization is not observed between different complexes. Relationships between flaviviruses are also demonstrated by less specific serological tests such as haemagglutination-inhibition (HI), complement-fixation and immunofluorescence assays with polyclonal antisera (Casals, 1957). The close relationship between the three viruses in the Tyuleniy complex (Table 1.1) is established by complement-fixation assays rather than neutralization (Casals, 1957). On the other hand, clear distinction between complexes is sometimes difficult when a particular member of a complex (e. g. Edge Hill virus of the Uganda S complex, Kokobera of the JE complex) shows a close relationship to another complex by criteria such as epitope analysis or RNA sequence homology (Calisher *et al.*, 1989).

TABLE 1.1
(adapted from Chambers *et al.*, 1990)

Antigenic complex[a] (number of members)	Type member or members with available sequence[b]	Arthropod vector[c]
Tick-borne encephalitis (12)	Central European encephalitis (TBE)	T*
	Far Eastern encephalitis (TBE-FE)	T
Rio Bravo (6)	Rio Bravo	U
Japanese encephalitis (10)	Japanese encephalitis (JE)	M
	Kunjin (KUN)	M
	Murray Valley encephalitis (MVE)	M
	St. Louis encephalitis (SLE)	M
	West Nile (WN)	M
Tyuleniy (3)	Tyuleniy	T
Ntaya (5)	Ntaya	M*
Uganda S (4)	Uganda S	M
Dengue (4)	Dengue type 1 (DEN-1)	M
	Dengue type 2 (DEN-2)	M
	Dengue type 3 (DEN-3)	M
	Dengue type 4 (DEN-4)	M
Modoc (5)	Modoc	U
Ungrouped (17)	Yellow fever (YF)	M*

[a] Antigenic groups are taken from Calisher *et al.*, 1989.

[b] Sequence data are avilable for JE, KUN, MVE, SLE, WN, DEN-1, DEN-2, DEN-3, DEN-4, TBE and YF (cited in the text).

[c] Arthropod vectors: T, tick; M, mosquito; U, unknown; *, arthropod vectors for some members of these groups have not been identified.

The JE complex (10 viruses) includes five members which are found in Australia. These are MVE, Kunjin virus (KUN), Alfuy virus (ALF), Kokobera virus and Stratford virus. Edge Hill virus in the Uganda S complex is also found in Australia but is not as closely related by serology to the rest of the Australian flaviviruses (Calisher *et al.*, 1989).

The two best studied flaviviruses in Australia, MVE and KUN, both utilize *Culex annulirostris* mosquito as the major arthopod vector; birds are the major vertebrate host (Kay, 1980; Marshall, 1979). Other viruses in the JE complex include Saint Louis encephalitis virus (SLE) and West Nile virus (WN), which also cycle between invertebrate (mosquito) and vertebrate hosts (mainly birds) (see review by Chamberlain, 1980).

1.2.2 Sequence homology

With the availability of nucleotide and protein sequence data for YF (Rice *et al.*, 1985) and ten viruses of the JE, DEN and TBE complexes (see below), it is found that homology at amino acid sequence level is always greater between members of the same antigenic complex than between viruses of different complexes. Therefore, the serological classification is generally in agreement with sequence homologies between flaviviruses (Mandl *et al.*, 1988; Mandl *et al.*, 1989a; Osatomi *et al.*, 1988; Pletnev *et al.*, 1990; Trent *et al.*, 1987).

Sequence data are available for the genomes of five viruses in the JE complex. These are MVE (Dalgarno *et al.*, 1986; Hahn *et al.*, 1987a; Lee *et al.*, 1990), WN (Castle *et al.*, 1985; Castle *et al.*, 1986; Castle and Wengler, 1987; Wengler and Wengler, 1981; Wengler *et al.*, 1985; Wengler and Castle, 1986), SLE (Trent *et al.*, 1987), JE (Hashimoto *et al.*, 1988; McAda *et al.*, 1987; Sumiyoshi *et al.*, 1986; Sumiyoshi *et al.*, 1987; Takegami *et al.*, 1986) and KUN (Coia *et al.*, 1988). Comparison of these five viruses provides an interesting contrast in protein sequence

homology: WN and KUN are the most homologous pair (93% amino acid sequence homology; Coia *et al.*, 1988); the next being JE and MVE (82%; Lee *et al.*, 1990). SLE is the least related in sequence to the above four viruses.

There is little variation in genome or protein sequence between different strains of the same flavivirus as shown by comparison of established genome sequence of two TBE subtypes (Mandl *et al.*, 1988), three DEN-2 strains (Hahn *et al.*, 1988) and two JE strains (McAda *et al.*, 1987; Sumiyoshi *et al.*, 1988): divergence in amino acid sequence between flavivirus strains is generally less than 5%. Comparison of genome sequences by oligonucleotide fingerprinting and restriction analysis of natural isolates of DEN-1 (Chu *et al.*, 1989), DEN-2 (Trent *et al.*, 1983), KUN (Lobigs *et al.*, 1986b) and MVE (Lobigs *et al.*, 1986a) also show small variation suggesting that flaviviruses are genetically stable relative to genomes of other RNA viruses such as influenza virus and polio virus which undergo rapid evolution (Holland *et al.*, 1982; Palese and Young, 1982). Partial sequence analysis of twelve strains of MVE from various parts of Australia and Papua New Guinea defined differences of between one and eleven amino acids in the envelope glycoprotein of 501 amino acids (Lobigs *et al.*, 1988). In contrast, MVE and its closest relative JE differ in 90 amino acids within the same protein.

1.3 Virion structure and genome organization

1.3.1 Virion structure

The virions of MVE, JE, DEN and WN as visualized by electron microscopy are small spherical particles of 40-50 nm in diameter (Filshie and Rehacek, 1968; Kitano *et al.*, 1974; Smith *et al.*, 1970; Wengler *et al.*, 1987). Three components make up the mature virion; they are the nucleocapsid (20-30 nm) and the envelope (≈40 nm in diameter) with surface projections which are 5-10 nm (Fig. 1.1).

The nucleocapsid is made up of capsid protein (C; 13-16K) and an RNA molecule which is the viral genome. The virus envelope is composed of a lipid bilayer generally

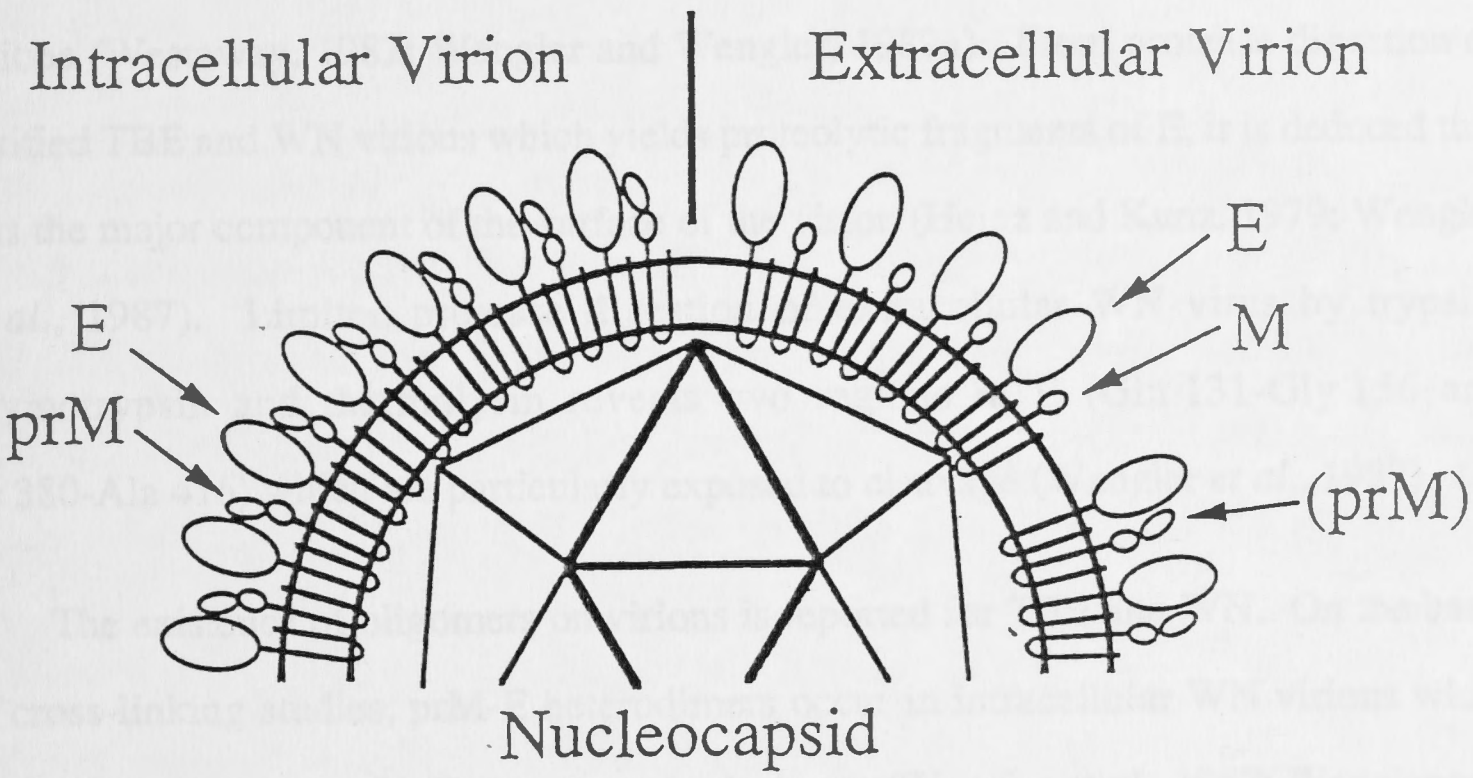


Figure 1.1 Flavivirus virion structure (adapted from Chambers *et al.*, 1990a). The nucleocapsid and the proteins in the envelope of flaviviruses are represented diagrammatically. The oligomeric association between envelope proteins (E, M and prM) in extracellular and intracellular WN is based on data from Wengler and Wengler (1989a).

assumed to derive from membranes of infected cells (see below). Virus-specific proteins which are associated with the envelope include the envelope protein (E; 51-60K), the matrix protein (M; 8-8.5K) and the pre-M protein (prM; 19-23K) (Fig. 1.1). E is the major component of the surface projections (Kitano *et al.*, 1974); M is a much smaller protein present on the envelope of extracellular virions; its glycoprotein precursor, prM, is present on the envelope of intracellular virions (Shapiro *et al.*, 1972; Wengler and Wengler, 1989a). Traces of prM are also detectable on extracellular virions (Westaway, 1987; Wengler and Wengler, 1989a). From protease digestion of purified TBE and WN virions which yields proteolytic fragments of E, it is deduced that E is the major component of the surface of the virion (Heinz and Kunz, 1979; Wengler *et al.*, 1987). Limited protease digestion of extracellular WN virus by trypsin, chymotrypsin and thermolysin reveals two regions in E (Gln 131-Gly 156 and Ile 380-Ala 416) which are particularly exposed to cleavage (Wengler *et al.*, 1987).

The existence of oligomers on virions is reported for TBE and WN. On the basis of cross-linking studies, prM-E heterodimers occur in intracellular WN virions while extracellular WN contains E monomers and trimers (Wengler *et al.*, 1987; Wengler and Wengler, 1989a). Hence, re-organization of the envelope proteins may occur before or during the release of virions, probably in close association with the conversion of prM to M. In contrast, extracellular TBE contains E monomers and dimers based on cross-linking studies (Heinz and Kunz, 1980a; 1980b). It has been suggested on the basis of differences between intracellular and extracellular virions that the rearrangement of oligomers on the surface of virions during maturation and release may influence infectivity of the virus (Wengler and Wengler, 1989a).

1.3.2 Structure of genome RNA

The RNA genome of flaviviruses consists of a single, plus-stranded RNA molecule (≈11 kb) which is infectious. A type 1 cap of the form m^7GpppAmp is present at the 5' terminus; there are no internal methylated adenine residues in

association with the cap structure (Cleaves and Dubin, 1979). It is generally accepted that mosquito-borne flaviviruses do not contain a 3'-terminal poly-A tract (Rice *et al.*, 1986). Instead, the 3' terminus (≈100 nucleotides) probably forms a conserved secondary structure involving complementary nucleotide sequences (Brinton *et al.*, 1986; Wengler and Castle, 1986; Hahn *et al.*, 1987a). However, certain strains of TBE appear to terminate with a 3'-poly-A tract while others have no poly-A at the 3' terminus (F. X. Heinz, personal communication). It has been proposed that the 5' terminus of the RNA may also form secondary structures and be involved in cyclization of the genome during infection (Brinton and Dispoto, 1988; Hahn *et al.*, 1987a).

1.3.3 Genome organization of flaviviruses

The establishment of genome sequence of eleven flaviviviruses including YF, viruses of the JE complex (see above), the four DEN subtypes (Biedrzycka *et al.*, 1987; Deubel *et al.*, 1986; Deubel *et al.*, 1988a; Gruenberg *et al.*, 1988; Hahn *et al.*, 1988; Mackow *et al.*, 1987; Mason *et al.*, 1987a; Osatomi *et al.*, 1988; Zhao *et al.*, 1986) and TBE (Mandl *et al.*, 1988; Mandl *et al.*, 1989a; Pletnev *et al.*, 1990; Yamshchikov and Pletnev, 1988) has revealed a common theme in organization: the occurrence of a single open reading frame (ORF; 10,158-10,299 nucleotides) which codes for 3386-3433 amino acids and spans at least 90% of the genome, and untranslated regions at the 5' and 3' termini. Viral proteins have been mapped on the genome by their N- and C-terminal sequences. The most complete mapping study has been for the KUN proteins (Speight *et al.*, 1988; Speight and Westaway, 1989a; 1989b). Terminal sequences of other flavivirus proteins have also mapped them on the genome (see review by Chambers *et al.*, 1990a). A common coding order of these viral proteins is established: the structural protein genes for C, prM/M and E occupy a quarter of the ORF at the 5' end, the rest of the ORF codes for the nonstructural proteins, which are only found in flavivirus-infected cells. The nonstructural proteins are named according to their order of appearance in the ORF, hence NS1 is followed by NS2A, NS2B, NS3, NS4A,

NS4B and NS5. Proteins NS1, NS3 and NS5 are identified and mapped for a number of flaviviruses, including MVE, YF and KUN (Lee *et al.*, 1990; Rice *et al.*, 1986b; Speight *et al.*, 1988). Idenfication of the other nonstructural proteins has been achieved only for KUN and YF (Speight *et al.*, 1988; Speight and Westaway, 1989a; Chambers *et al.*, 1989; see below).

Fig. 1.2 presents the MVE genome organization based on the genome sequence (Dalgarno et al., 1986; Hahn *et al.*, 1987a; Lee *et al.*, 1990; A. Nestorowicz, personal communication), terminal sequences of NS1, NS3 and NS5 and homology with the genome organization of KUN and YF (see above). The genome (11,013 nucleotides) contains an ORF of 10,302 nucleotides flanked by a 5' untranslated region (≈95 nucleotides) and a 3' untranslated region (616 nucleotides). Proteins encoded in the ORF are in the same order as those of other flaviviruses.

1.3.4 Flavivirus-encoded proteins

Structural proteins

Structural proteins C, E, M and its precursor prM occur in virions (see above) and virus-infected cells. Protein C and the genome RNA form the nucleocapsid. There is little conservation in C protein sequences between flaviviruses of different complexes (Trent *et al.*, 1987). However, there is always a high proportion of basic amino acids (Arg and Lys) in the protein consistent with a role in capsid formation (see review by Rice *et al.*, 1986a). Intracellular and virion forms of C proteins have been shown to differ in mobility by SDS-PAGE (Rice *et al.*, 1986a; Chambers *et al.*, 1990a). The virion C protein of WN and KUN lacks a hydrophobic peptide which is at the C-terminus of the predicted protein sequence (Nowak *et al.*, 1989; Speight and Westaway, 1989b). It is has been proposed that the intracellular C protein with the hydrophobic sequence is membrane-associated and is converted to the virion C protein by removal of the hydrophobic C-terminus (see below).

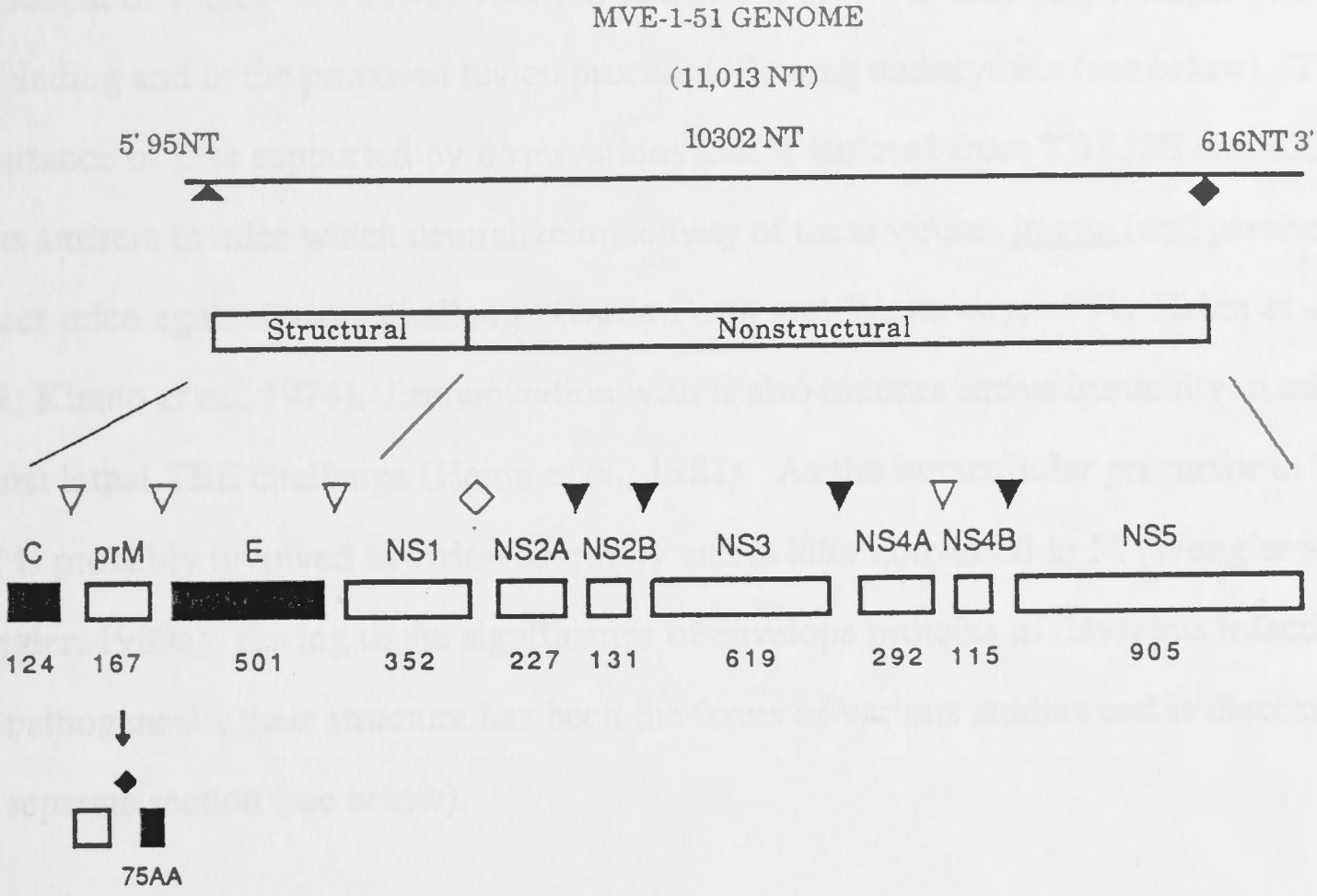


Figure 1.2 Genome organization of MVE. The RNA genome of MVE-1-51 (prototype strain) is represented by the solid line. The single long open reading frame (ORF) which spans the genome begins with an AUG (solid triangle) and terminates in an UAA (solid diamond). The polyprotein encoded by the ORF is represented by the open bar. Viral proteins (structural and nonstructural) produced by proteolytic processing of the polyprotein are shown below, the lengths in amino acids are indicated. Cleavage specificity is indicated as follows: open triangles indicate signalase-like cleavage sites following a hydrophobic sequence and a short side chain amino acid; arrowheads indicate cleavage after two basic residues; the open diamond indicates cleavage after a consensus tripeptide Val-X-Ala without a preceding hydrophobic sequence; the arrow indicates cleavage after the consensus sequence Arg-X-Arg/Lys-Arg. This genome organization is deduced from the MVE genome sequence (Dalgarno *et al.*, 1986; Hahn *et al.*, 1987a; Lee *et al.*, 1990; A. Nestorowicz, personal communication) on the basis of data on genome organization of YF and KUN (Chambers *et al.*, 1990b; Speight *et al.*, 1989). Positions of small nonstructural proteins NS2A, NS2B, NS4A and NS4B are tentative and based on homology with KUN proteins (Speight *et al.*, 1988; Speight and Westaway, 1989a).

The envelope proteins E,. prM and M may assume important roles during the initiation of flavivirus infection and in assembly of infectious virions. As the dominant component of spikes on virus surface (Kitano *et al.*, 1974), E may play a major role in cell binding and in the proposed fusion process following endocytosis (see below). The importance of E is supported by observations that E isolated from TBE, JE and KUN elicits antisera in mice which neutralize infectivity of these viruses in vitro and passively protect mice against virus challenge (Della-Porta and Westaway, 1977; Heinz *et al.*, 1981; Kitano *et al.*, 1974). Immunization with E also induces active immunity in mice against lethal TBE challenge (Heinz *et al.*, 1981). As the intracellular precursor to M, prM is probably involved in virion assembly and is later converted to M (Wengler and Wengler, 1989a). Owing to the significance of envelope proteins in flavivirus infection and pathogenesis, their structure has been the focus of various studies and is discussed in a separate section (see below).

Large nonstructural proteins

Three large nonstructural proteins NS1 (41-49K), NS3 (67-76K) and NS5 (91-98K) have been identified in flavivirus-infected cells. These proteins are hydrophilic by comparison with the smaller nonstructural proteins. Comparison of the molecular weights (mol. wts.) and protein sequences between flaviviruses has shown that these proteins are strongly conserved (Lee *et al.*, 1990; Rice *et al.*, 1986a).

Various forms of NS1 protein including NS1 dimers (Winkler *et al.*, 1988; 1989), a larger NS1 protein containing part of NS2A (Mason *et al.*, 1987b; Mason, 1989) and a soluble complement fixing antigen (Smith and Wright, 1985; Lee *et al.*, 1989; Winkler *et al.*, 1989) have been observed. NS1 is distinguished from other nonstructural proteins by the conservation of positions of 11 cysteines (Rice *et al.*, 1986a), glycosylation (see below) and its cell surface distribution (Schlesinger *et al.*, 1990). It is likely that the conserved cysteines are involved in disulfide bonds, thus maintaining NS1 in a conserved conformation reminiscent of the structural proteins (see below).

NS1 undergoes Asn-linked glycosylation in various types of cells (Lee *et al.*, 1989; Mason, 1989). The positions of the tripeptides Asn-X-Thr/Ser which are potential Asn-linked glycosylation sites (Kornfeld and Kornfeld, 1985) are conserved in the JE complex and in the four DEN subtypes (see Chambers *et al.*, 1990a). Complex glycans are attached to NS1 of JE in the Golgi cisternae and are associated with the secreted forms of NS1 which appear simultaneously with extracellular virus (Mason, 1989). Based on the above observations on structure, glycosylation and cellular distribution of NS1, it has been suggested (Winkler *et al.*, 1989; Mason, 1989; Lee *et al.*, 1989) that NS1 may have roles in virus assembly or release. NS1 protects animals from YF and DEN-2 challenge by active immunization (Schlesinger *et al.*, 1985; 1987) and elicits antisera which can be used to passively protect animals against lethal challenge by DEN-2 and YF (Brandriss and Schlesinger, 1984; Schlesinger *et al.*, 1985). Monoclonal antibodies (mAbs) against DEN-2 NS1 also passively protect mice (Henchal *et al.*, 1988).

The NS3 and NS5 proteins do not contain extensive hydrophobic regions and are both strongly conserved. It has been suggested that these are cytoplasmic proteins which are involved in replication of the RNA genome. Conserved sequence motifs in the N- and C-terminal portions of NS3 show resemblance to the active domains of the serine protease and the helicase enzymes respectively (Bazan and Fletterick, 1989; Gorbalenya *et al.*, 1989). Studies of in vitro translation of NS2B-NS3 genes have shown that autoproteolytic activity is associated with the N-terminal region of NS3 (Chambers *et al.*, 1990c). Thus NS3 may have a dual role in proteolytic processing and RNA replication. Conserved sequence motifs in NS5 are homologous to the RNA-dependent RNA polymerase of eukaryotic viruses indicating that NS5 probably acts as the viral polymerase (Rice *et al.*, 1986a).

Small nonstructural proteins

Four smaller proteins NS2A (19K), NS2B (10K), NS4A (13K) and NS4B (21K) are identified in KUN-infected cells (Speight *et al.*, 1988; Speight and Westaway, 1989a). NS2A (20K), NS2B (15K) and NS4B (27K) have also been identified for YF (Chambers *et al.*, 1989). These proteins present problems in identification owing to their small size, quantity and variable mobility by SDS-PAGE (Speight *et al.*, 1988; Chambers *et al.*, 1989). The sequences of NS2A, NS2B, NS4A and NS4B are hydrophobic by comparison with the large nonstructural proteins and show poor homology between flaviviruses. It appears that these proteins do not have enzymic roles but may be required for their hydrophobic properties (see below).

1.4 Replication of flaviviruses in tissue culture

Flaviviruses are able to infect a wide range of tissue culture cell lines including lines of mammalian, avian and insect origin (Brinton, 1986). There may be differences in virus growth and yield as well as in the extent of damage to host cells; however, marked inhibition of host macromolecular synthesis is generally not observed (Westaway, 1987). The mosquito-borne flaviviruses (YF, DEN subtypes and JE serocomplex) often generate cytopathic effects and are commonly cytocidal for vertebrate cells. Persistent infection occurs more frequently in mosquito cells and little cytopathic effect is observed (Brinton, 1986). Persistent infection of mammalian cells has been reported for JE and WN (Schmaljohn and Blair, 1977; Brinton *et al.*, 1985).

Several features are consistent during flavivirus infection. These include the growth curve which shows a latent period of 12-16 hours (Mussgay *et al.*, 1975) and the proliferation of membranes in cytoplasm including smooth and rough endoplasmic reticulum (RER) (Westaway, 1987).

1.4.1 Initiation of infection

Studies by electron microscopy of WN-infection of a macrophage cell line, P388D1, show that single virions or aggregates appear in clathrin-coated pits on the cell surface before rapid uptake (Gollins and Porterfield, 1985) consistent with entry by receptor-mediated endocytosis (see review by Marsh and Helenius, 1989). Subsequent to entry, WN appears in endosomes where fusion between virus envelope and endosomal membrane occurs at acidic pH causing the release of nucleocapsids into the cytoplasm (Gollins and Porterfield, 1985; 1986). This acid-induced fusion process is inhibited by weak bases such as ammonium chloride and chloroquin (Gollins and Porterfield, 1985). Anti-WN rabbit IgG inhibits fusion in a concentration-dependent manner parallel to its ability to neutralize virus infectivity in vitro, this indicates that neutralization may occur by inhibition of the fusion process (Gollins and Porterfield, 1986). KUN and YF also adsorb to coated pits on Vero cell surface and appear in coated vesicles consistent with uptake by receptor-mediated endocytosis (Ng and Lau, 1988; Ishak *et al.*, 1988). Enhancement by anti-viral antibodies occurs in the P388D1 cell line, whereby the uptake of WN virions shows at least a nine-fold increase in efficiency in the presence of subneutralizing amounts of polyclonal or monoclonal antibodies (Gollins and Porterfield, 1984; 1985). The Fc receptor on the surface of these cells is thought to be responsible for the enhancement; however, the same pathway of endocytosis and fusion appears to be utilized as in the absence of antibody (Gollins and Porterfield, 1985).

A conflicting observation has been reported for the entry of DEN-2 into C6/36 mosquito cells and human monocytes, and the entry of JE into C6/36 cells: electron microscopy shows direct penetration of the plasma membrane with no evidence of receptor-mediated endocytosis (Hase *et al.*, 1989a; Hase *et al.*, 1989b). It is not clear if productive infection follows such a route of entry.

1.4.2 Translation of genome RNA

After uncoating of the nucleocapsid, the genome RNA is released into the cytoplasm and translated into viral proteins. The mechanism of uncoating is not known. From immunofluorescence studies which show that accumulation of viral proteins occurs primarily in the perinuclear region in the cytoplasm, the site of viral protein translation is deduced to be cytoplasmic and is probably assocated with the perinuclear RER (Ng *et al.*, 1983; Westaway and Goodman, 1987).

Translation of the genome RNA is thought to produce a high mol. wt. polyprotein which gives rise to individual proteins by co- and post-translational processing. Several lines of evidence support this including the existence of a single long ORF and the detection of high mol. wt. precursors in KUN-infected Vero cells and DEN-2 infected hamster kidney cells (Cleaves, 1985; Crawford and Wright, 1987); the former were incubated with a leucine analogue to inhibit signalase cleavage and the latter were pulse-labelled with S^{35}-methionine. Identification of the proteases and other enzymes responsible for processing has been achieved for some viral proteins (see below).

Translation of the flavivirus genome begins at the first AUG initiation codon in the ORF as shown from the N-terminal sequence of the C proteins of YF, SLE, WN, KUN, JE, TBE and DEN-3 (Bell *et al.*, 1985; Castle *et al.*, 1985; Coia *et al.*, 1988; Sumiyoshi *et al.*, 1987; Boege *et al.*, 1983; Osatomi *et al.*, 1988). However, additional forms of WN and DEN-3 C proteins derive from initiation at the second AUG codon in the ORF (Castle *et al.*, 1985; Osatomi *et al.*, 1988). Termination of translation is assumed to occur at the first stop codon in the ORF; there appears to be no preference for a particular termination codon as UAA, UGA and UAG are all used (see review by Chambers *et al.*, 1990a). The sequence of appearance of viral proteins has been determined by translation mapping experiments using KUN-infected Vero cells and generally follows the order of proteins encoded in the RNA genome (Schrader and Westaway, 1988). However, this study has revealed anomalies in the synthesis of

KUN NS5 protein prompting suggestions that an alternative mechanism such as internal initiation of translation may also occur (Schrader and Westaway, 1988).

1.4.3 Proteolytic cleavage

Proteolytic cleavage sites and the identity of proteases involved in processing flavivirus polyproteins are predicted from deduced amino acid sequence data and direct N-terminal sequence analyses. At least four types of cleavage events occur based on cleavage specificities (see Fig. 1.2). Cleavage sites at the N- and C-termini of prM and E and at the N-terminus of NS4B are preceded by hydrophobic signal peptides and occur after small amino acids, consistent with signalase cleavage; the tripeptide Val-X-Ala quite often precedes the cleavage sites (see Chambers *et al.*, 1990a). Supporting evidence comes from cell-free translation of DEN-2, WN and YF genomes which demonstrates the requirement for a microsomal membrane fraction for cleavage of polyprotein precursors into C, prM and E in the absence of the nonstructural proteins (Markoff, 1989; Nowak *et al.*, 1989; Ruiz-Linares *et al.*, 1989). Expression of the structural proteins of DEN-2, DEN-4 and JE using vaccinia virus and baculovirus vectors also demonstrates cleavage of polyprotein precursors into prM and E in the absence of nonstructural proteins (Deubel *et al.*, 1988b; Matsuura *et al.*, 1989; Zhao *et al.*, 1987; Zhang *et al.*, 1988).

Cleavage at the C-terminus of NS1 is preceded by the tripeptide Val-X-Ala without a preceding hydrophobic tract. As NS1 is probably translocated into the lumen of the RER by the signal peptide at the C-terminus of E, this cleavage probably occurs in the lumen of the RER. Both JE- and YF-infected cells produce an additional form of NS1 which contains a portion of the adjoining NS2A protein (Mason *et al.*, 1987b; Chambers *et al.*, 1990b) indicating inefficient cleavage at the C-terminus of NS1.

The nonstructural proteins NS2B, NS3 and NS5 are generated by cleavage after two basic residues. From the absence of hydrophobic signal peptides, NS2B, NS3 and

NS5 are deduced to be cytoplasmic proteins, thus the cleavage probably occurs in the cytoplasm (Speight *et al.*, 1988). A virus-encoded protease has been proposed to be responsible for this cleavage (Rice *et al.*, 1985). A possible candidate is the NS3 protein which possesses sequences homologous to serine proteases (Bazan and Fletterick, 1989; Gorbalenya *et al.*, 1989). Two basic residues also precede the cleavage site which converts the putative anchored C protein into the anchorless form found in KUN and WN virions (by removing the hydrophobic C-terminus; Nowak *et al.*, 1989; Speight and Westaway, 1989).

Cleavage at the N-terminus of M follows the sequence Arg/Lys-Arg-X-Arg/Lys-Arg (Chambers *et al.*, 1990a) and converts the intracellular precursor prM to M in extracellular virions. This conversion occurs late in infection, probably during or before virus release as intracellular virus has the prM protein (Wengler and Wengler, 1989a). This cleavage is inefficient as prM is still detectable in released flaviviruses (Wengler and Wengler, 1989a; Westaway, 1987). Cleavage probably occurs in the lumen of the Golgi cisternae where an enzyme with the appropriate cleavage specificity exists (see review by Strauss and Strauss, 1985).

1.4.4 RNA synthesis

A generally accepted scheme for genome RNA replication during flavivirus infection is that the genome RNA is the template for synthesis of full length minus strands which are then transcribed into genome length plus strands (Westaway, 1987). It is expected that transcription is regulated to produce more plus strand than minus strand RNAs for translation of viral proteins and virus assembly. It has been speculated that the association between C and plus strand RNA may prevent generation of the minus strand and initiate encapsidation (Rice *et al.*, 1986a).

RNA species in infected cells have been described for KUN, DEN-2 and WN (Chu and Westaway, 1985; Cleaves *et al.*, 1981; Wengler and Wengler, 1981). There

are two major forms in addition to the full length plus and minus strand RNAs; they are the replicative intermediates (RI) and replicative forms (RF). RFs are double stranded RNAs and RIs are nascent single stranded RNAs in association with template, single stranded RNAs (Westaway, 1987). From incorporation of radio-label and RNAse resistance, RIs and RFs are intermediates in viral RNA replication which occurs by a semi-conservative mechanism (Westaway, 1987).

The polymerase complex can be isolated from infected cells in the heavy membrane fraction (Grun and Brinton, 1988; Westaway, 1987); however, the composition of these complexes is still unresolved. NS3 and NS5, the two possible components of the polymerase complex, have been localized by immunofluorescence using protein-specific antibodies to the perinuclear membranes where virus-specific RNAs accumulate and are detected by anti-dsRNA antibodies (Ng *et al.*, 1983; Westaway, 1987). It appears that the replication of flavivirus genome RNA occurs near the cellular membranes; the association of the polymerase complex with membranes suggests that hydrophobic viral proteins (such as the small nonstructural proteins) and/or host factors may also be components in the polymerase complex (Grun and Brinton, 1988).

1.4.5 Assembly and release of virions

The assembly of virion particles involves the genome RNA, C, and envelope proteins prM and E. Morphological studies by electron microscopy show virions predominantly in the lumen of the RER (Filshie and Rehacek, 1968; Hase *et al.*, 1987a; Ishak *et al.*, 1988; Leary and Blair, 1980). In some studies (Hase *et al.*, 1987a; Ishak *et al.*, 1988; Leary and Blair, 1980), virus is also observed in the Golgi cisternae or cytoplasmic vacuoles. Based on the above observations and the perinuclear localization of prM and E (Westaway and Goodman, 1987), it has been proposed (Hase *et al.*, 1987a; Leary and Blair, 1980) that envelopment occurs at the perinuclear membranes; enveloped virions probably enter the lumen of the RER in the process and undergo further processing before release from cells. Questions regarding the site of

encapsidation remain unresolved. As naked nucleocapsids or incomplete virions have not been observed in those morphological studies which show virions in the lumen of the RER, it seems likely that encapsidation and envelopment occurs concurrently at the same location. The intracellular form of C containing a hydrophobic membrane anchor may be involved in the encapsidation process since the newly formed nucleocapsids may contain this anchored C and be close to the ER membranes where envelopment occurs (Nowak *et al.*, 1989). The conversion of anchored C to the virion-associated anchorless form probably occurs during or after envelopment (Nowak *et al.*, 1989).

A different pathway of assembly and release of virion is suggested in a study by electron microscopy of DEN-2-infected C6/36 cells: naked nucleocapsids appear in the cytoplasm and bud into vacuoles or through the plasma membrane into the extracellular space (Hase *et al.*, 1987b). By comparing this study and a separate study of JE-infected C6/36 cells (Hase *et al.*, 1987a) which shows virion assembly and release by the pathway described in the previous paragraph, Hase *et al.*, (1987a) proposed that different mechanisms for virion assembly may exist for flavivirus infection.

1.5 Structure of the flavivirus envelope proteins

1.5.1 Structural properties

The M protein: The M protein is approximately 8K (75 amino acids for all flaviviruses) and is only detected on extracellular virus (Shapiro *et al.*, 1972; Russell *et al.*, 1980). The intracellular precursor, prM, is 19-23K (166-168 amino acids). The Asn-X-Thr/Ser tripeptide occurs only in the 'pr' segment, consistent with the glycosylation of prM but not M (Chambers *et al.*, 1990a). The prM proteins of WN, KUN, DEN-2 and DEN-4 contain 'mannose-rich' glycans (see Chambers *et al.*, 1990a), indicating processing of prM in the lumen of the RER. There are six strictly conserved cysteines in the 'pr' segment of prM of all flaviviruses; these may be involved in disulfide bonds which contribute to the conformation of prM. There is no conserved cysteine in M. Other than the conserved cysteines, the prM protein, including the M

sequence appears not to be conserved in sequence. The hydropathy pattern for prM reveals that the 'pr' segment is hydrophilic. M contains a hydrophilic region in the N-terminal half; the C-terminal half comprises two hydrophobic tracts which are interrupted by a charged residue. The first hydrophobic tract may act as a 'stop transfer' sequence while the latter is possibly a signal peptide for E (Markoff, 1989). The role of the prM and M proteins is not clearly defined. The heterodimers of prM-E in intracellular WN suggests a close association of prM and E during assembly (Wengler and Wengler, 1989a). A conserved conformation of prM based on possible conservation of disulfide bonds may be important to this association.

The E protein: The E proteins of flaviviruses is 51-60K (493-501 amino acids). Fig. 1.3 shows the sequence for the MVE E protein and a co-listing of E sequences for nine other flaviviruses. The most conserved feature in these sequences is the occurrence of twelve cysteines at identical positions in E of all flaviviruses. These cysteines are all involved in disulfide bonds in the WN E protein at Cys 3-30, Cys 60-121, Cys 74-105, Cys 92-116, Cys 186-284 and Cys 301-332 (Nowak and Wengler, 1987). These disulfide bonds are likely to be strictly conserved and thus be important in maintaining the conformation of E. The pattern of disulfide linkages in WN E protein provides valuable data on the two-dimensional structure of E (Nowak and Wengler, 1987) which probably applies to all flaviviruses (see below). Other than the cysteines, strongly conserved sequences occur in the first 40 amino acids at the N-terminus, residues 100-110 and in the 40 amino acids after residue 420. The sequence from Asp 98 to Gly 111 is the most conserved with only one difference in ten flaviviruses (see below). By contrast, the region between residues 120 and 210 is highly variable between flaviviruses.

The hydropathy pattern for E shows strong conservation between flaviviruses (McAda *et al.*, 1987). The C-terminal 39 amino acids of all E proteins comprise two uncharged tracts separated by one or three charged residues. Apart from acting as the

Figure 1.3 Co-listing of E protein sequences of ten flaviviruses. The E protein sequences of nine flaviviruses, JE (Sumiyoshi *et al.*, 1988), KUN (Coia *et al.*, 1988), WN (Wengler *et al.*, 1985), SLE (Trent *et al.*, 1987), YF (17D vaccine strain; Rice *et al.*, 1985), DEN-1 (Mason *et al.*, 1987a), DEN-2 (Jamaica genotype; Deubel *et al.*, 1986), DEN-4 (Zhao *et al.*, 1986) and TBE (Western subtype; Mandl *et al.*, 1988), are aligned to that of MVE (Dalgarno *et al.*, 1986) and are listed below the MVE E sequence with dots indicating identical amino acids. Hyphens indicate gaps inserted to maximize homology. The twelve conserved cysteines are indicated by asterisks, the Asn-X-Thr/Ser glycosylation sites are underlined and the hydrophobic tracts at the C-terminus are overlined.

```
         *        |        |        *        |        |        *        |   *    |        90
MVE      FNCLGMSSRDFIEGASGATWVDLVLEGDSCITIMAADKPTLDIRMMNIEATNLALVRNYCYAATVSDVSTVSNCPTTGESHKTKRADHNY
JE       ......GN....................L...N......V..I....SQ..E..S...H.S.T.I...AR......A.VE....SS.
KUN      .......N...L..V.............V...SK....I.VK...M..A...E..S...L.T..EL..KAA...M..A.ND....PSF
WN       .......N...L..V.............V...SK....I.VK...M..A...D..S...L.S...L..RAA...M..A.NE....PAF
SLE      .....T.N...V........I......G..V.V..PE.....FKV.KM...E..T..K...E..LDTL...AR......A.N...S.PTF
YF-17D   AH.I.ITD......VH.G...SAT..Q.K.V.V..P...S...SLETVAIDRP.E..KV..N.VLTH.KINDK..S...A.LAEENEGDN
DEN-1    MR.V.IGN...V..L.......V...HG..V.T..K.......ELLKT.V..P.VL.KL.IE.KI.NTT.D.R...Q..ATLVEEQ.T.F
DEN-2    MR.I.I.N...V..V..GS...I...HG..V.T..KN.....FELIKT..KQP.TL.K..IE.KLTNTT.E.R...Q..PSLNEEQ.KRF
DEN-4    MR.V.VGN...V..V..GA.......HGG.V.T..QG.....FELTKTT.KEV..L.T..IE.SI.NIT.ATR...Q..PYLKEEQ.QQ.
TBE      SR.THLEN...VT.TQ.T.R.T....LGG.V..T.EG..SM.VWLDA.YQE.P.KT.E..LH.KL..TKVAAR...M.PATLAEEHQGGT

         *        |   *    |   *    |*       |        |        |        |        |        180
MVE      LCKRGLTDRGWGNGCGLFGKGSIDTCAKFTCSNSAAGRLILPEDIKYEVGVFVHGSTDSTSHGNYSTQIGANQAVRFTISPNAPAITAKM
JE       V..Q.F......................S.TSK.I..T.Q..N......I....T.T.EN.....A.V..S..AK...T....S..L.L
KUN      V..Q.VV.....................A..TK.T..T..K.N.....AI....P.TVE.....F..T..A..G..S.T.A..SY.L.L
WN       V..Q.VV.....................A.TTK.T.WI.QK.N.....AI....P.TVE...K----...T..G..S.T.S..SY.L.L
SLE      V...DVV.......................K.K.T.KT..R.N.....AI..............E...K...A......Q..SF..N.
YF-17D   A...TYS...............VA......AK.MSLFEVDQTK.Q.VIRAQL.VGAKQENWNTDIK--------TLKFDALSGSQEVEF
DEN-1    V.R.TFV...............LI.....K.VTKLE.KIVQY.NL..S.I.T..TGDQHQVGNETTEHG-----TTA..T.Q..TSEIQL
DEN-2    ...HSMV..............G.V...M...KKNME.KVV...NLE.TIVITP.SGEEHAVGNDTGKHG-----KEIK.T.QSSITE.EL
DEN-4    I.R.DVV..............GVV.....S..GKIT.N.VRI.NLE.T.V.T..NGDTHAVGNDT.NHG-----.TAM.T.RS.SVEV.L
TBE      V...DQS.....H.........VA.V.AA.EAKKKATGHVYDAN.IVYT.K.EPH.GDYVAA.ETHSGRKTA--S....SEKTIL.--.

                *         |                |               |        |          |        |       261
MVE      GDYGEVTVECEPRSGLNTEAYY------VMTIGTKHFLVHREWFNDLLLPWTSPASTE---WRNREILVEFEEPHATKQSVVALGSQEGA
JE       .......LD...........F.------...V.S.S........H..A......S..A---.....L.M...A...............G
KUN      .E......D......ID.S..------...V............M..N...S.AE.NV---.....T.M..........I.......
WN       .E......D......ID.S..------..SV.E.S........M..N...S.AG..T---.....T.M...................
SLE      .E..T..ID..A...I...D..------.F.VKE.SW..N.D..H..N.......T.D---.....T...........T........R
YF-17D   IG..KA.L..QVQTAVDFGNS.------IAEME.ESWI.D.Q.AQ..T...Q.GSGGV---..EMHH.....P...ATIR.L...N...S
DEN-1    T...AL.LD.S..T..DFNEMV------LL.MEK.SW...KQ..L..P.....G...SQET.NRQDL..T.KTA..K..E..V.......
DEN-2    TG..T..M..S..T..DFNEMV------LLQMED.AW....Q..L..P...LPG.D.QGSN.IQK.T..T.KN...K..D..V.......
DEN-4    P....L.LD...GLE.YFNEMI------L.KMKK.TW...KQ..LN.P....AG.D.S.VH.NYK.RM.T.KV...KR.D.TV.E.....
TBE      .E..D.SLL.RVA..VDLAQTVILELDKTVEHLPTAWQ...D.....A...KHEFAQN---.N.A.R....GA...V.MD.YN..D.T.V

                 |               |        *|         |     *    |         |          |   *   |  347
MVE      LHQALAGAIP---VEFSSSTLKLTSGHLKCRVKMEKLKLKGTTYGMCTEKFTFSKN-PADTGHGTVVLELQYTGSDGPCKIPISSVASLN
JE       .........V---..Y..-SV.........L..D..A..........S.A..-..........I..S.S.........V......
KUN      .........---.....N.V...............Q.......V.SKA.R.LGT-.................T...............
WN       .........---.....N.V...............Q.......V.SKA.K.ART-.................T.....V.........
SLE      PAT.......---ATV.....T.Q.......A.LD.V.I.......DSA......-.T.......IV.......N...RV...VT.N.M
YF-17D   .KT..T..MRVTKDTNDNNLY..HG..VS....LSA.T....S.KI..D.MF.V..-.T........MQVKVSKGA-..R..VIVADD.T
DEN-1    M.T..T..TE----IQT.G.TTIFA......L..D..T...MS.V...GS.KLE.E-V.E.Q....LVQVK.E.T.A.....F..QD-EK
DEN-2    M.T..T..TE----IQM..GNL.FT......LR.D..Q...MS.S...G..KIV.E-I.E.Q...I.IRV..E.DGS.....FEIMD-.E
DEN-4    M.S.....TE----VD.GDGNHMFA.....K.R....RI..MS.T..SG..SID.E-M.E.Q...T.VKVK.E.AGA...V..EIRD-V.
TBE      .LK....VP.---AHIEGTKYH.K...VT.E.GL....M..L..T..DKTKFTW.RA.T.S..D...M.VTFS.TK-..R..VRA..HSP

                |   |              |           |           |           |           |           437
MVE      DMTPVGRMVTANPYVASSTANAKVLVEIEPPFGDSYIVVGRGDKQINHHWHKEGSSIGKAFSTTLKGAQRLAALGDTAWDFGSVGGVFNS
JE       .......L..V..F..T.S..S.....M..........................A..TL.........................I......
KUN      .L.....L..V..F.SV........I.L..............EQ........S........TA...........................T.
WN       .L.....L..V..F.SVA...S...I.L..............EQ........S........T...R........................T.
SLE      .L.....L..V..FISTGG..N..MI.V..............TT...Y............LA..W.......V..........I......
YF-17D   AAINK.IL..V..IASTNDDE--..I.VN........I.....SRLTYQ..........L.TQ.M..VE...VM.......S.A..F.T.
DEN-1    GV.QN..LI....I.TDKEKP--.NI.A.....E......A.E.ALKLS.F.K......M.EA.AR..R.M.I..........I....T.
DEN-2    KRHVL..LI.V..I.TEKDSP--.NI.A.........II.VEPG.LKLN.F.K.....QM.E..MR..K.M.I..........L....T.
DEN-4    KEKV...IISST.LAENTNSV--TNI.L.R.-L.....I.V.NSALTL..FRN......M.E..YR..K.M.I..E..........L.T.
TBE      .VNVAM-LI.P..TIENNGGG--.FI.MQL.P..NI.Y..----ELS.Q.FQK.....RV.QK.K..IE..T.I.EH......A..FLS.

          |          |          |         |          |         |          501
MVE      IGKAVHQVFGGAFRTLFGGMSWISPGLLGALLLWMGVNARDKSIALAFLATGGVLLFLATNVHA
JE       .........................TQ..M..............R.............V........
KUN      V...I.........S........TQ...........I....R....T...V........SV....
WN       V...I.........S........TQ...........I....R...MT...V........SV....
SLE      .........................TQ...........LQ...R..S.TL..V..I.I....S.Q.
YF-17D   V..GI.T...S..QG....LN..TKVIM..V.I.V.I.T.NMTMSMSMILV.VIMM..SLG.G.
DEN-1    V..LI..I..T.YGV..S.V..TMKIGI.I..T.L.L.S.ST.LSMTCI.V.M.T.Y.GVM.Q.
DEN-2    ....L.....AIYGAA.S.V..TMKI.I.VIIT.I.M.S.ST.LSVSLVLV.V.T.Y.GAM.Q.
DEN-4    L.........SVYT.M...V..MIRI.I.F.V..I.T.S.NT.M.MTCI.V..IT...GFT.Q.
TBE      ......T.L....NSI...VGFLPKL...VA.A.L.L.M.NPTMSMS..LA..LV.AMTLG.G.
```

membrane anchor, it has been proposed that the first uncharged tract acts as a stop transfer sequence and the second as the signal peptide for NS1 (Ruiz-Linares *et al.*, 1989). There is no other extensive hydrophobic region in E. Based on proteolytic digestion of TBE and WN (Heinz and Kunz, 1979; Wengler *et al.*, 1987), it appears that the ectodomain occupies most of E except for the hydrophobic C-terminus. There are regions in WN E protein particularly exposed to proteolytic cleavage (see above).

The E proteins of flaviviruses are generally glycosylated. The Asn-linked glycosylation site (Asn-X-Thr/Ser) occurs in the E sequences of most flaviviruses except WN and KUN (see below). The glycosylation site at Asn 154 is in seven of eleven flaviviruses including MVE, JE, SLE, TBE and the four dengue subtypes (Chambers *et al.*, 1990a). The dengue viruses all contain additional glycosylation sites at Asn 67. The absence of glycosylation site in the WN E protein corresponds with the lack of glycosylation (Wengler *et al.*, 1985). For the E proteins of the 17D and Asibi strains of YF (Hahn *et al.*, 1987b; Rice *et al.*, 1985), the glycosylation sites are an Asn-Met-Thr in the hydrophobic C-terminus and an Asn-Pro-Thr in the ectodomain. In view of the absence of glycans on some strains of YF (Schlesinger *et al.*, 1983), these sites, which are probably not good acceptor sites for glycosylation (Kornfeld and Kornfeld, 1985), may affect the pattern of glycosylation of YF E protein. Glycosylated forms of KUN E have been identified in infected cells (Wright *et al.*, 1981), even though the KUN strain used for sequencing does not contain Asn-X-Thr/Ser in E and the E in KUN virion is apparently not glycosylated (Wright, 1982). Factors such as strain variation and utilization of tripeptides such as Asn-X-Cys (Kornfeld and Kornfeld, 1985) may account for this phenomenon.

The composition of the glycans in DEN-2, JE and TBE E has been studied using radio-labelled sugars and specific endoglycosidases. Glycans of either the high-mannose or the complex type are attached to E of these viruses (Mason, 1989; Smith and Wright, 1985; Winkler *et al.*, 1987). Studies of JE-infected C6/36 and Vero cells

(Mason, 1989) have shown that the cell associated E contains a single mannose-rich glycan while extracellular virus associated E has a complex glycan. It has been suggested that the glycans are converted to complex forms in the Golgi cisternae before or during release (Mason, 1989). Tunicamycin treatment which inhibits Asn-linked glycosylation impairs the release of virion-associated E but does not abolish it (Mason, 1989). Deglycosylation of TBE using endoglycosidase F has no effect on its infectivity, haemagglutination activity or reactivity with either polyclonal sera or mAbs defining eight epitopes on E (Winkler *et al.*, 1987). Therefore, it appears that glycosylation does not affect the critical functions of E although subtle effects on maturation and release of virus cannot be ruled out.

1.5.2 Epitopes in the envelope proteins

MAbs directed against E show different degrees of cross-reaction with other flaviviruses and their biological activities include neutralization, HI and complement-fixation. It is not uncommon for anti-E mAbs to have more than one of these activities (see below). Despite reactivity with virus, anti-M mAbs have not been reported to possess these biological activities (see review by Heinz, 1986). Characterization of mAbs and epitope mapping studies have been used to determine the antigenic structure of E and to locate regions of functional significance in the E proteins of MVE, SLE, JE, TBE, DEN-2 and YF (Cammack and Gould, 1986a; Cecilia *et al.*, 1989; Guirakhoo *et al.*, 1989; Hawkes *et al.*, 1988; Henchal *et al.*, 1985; Roehrig *et al.*, 1983).

MAbs against MVE have been characterized (Hawkes *et al.*, 1988), these define eight epitopes in E and show different properties of ELISA cross-reactivities, neutralization and HI activities. Topological studies by competitive binding assays (CBAs) using these mAbs showed that they define a continuum of overlapping epitopes (J. T. Roehrig, personal communication). MVE and other viruses of the JE complex share extensive homology in E sequence. It is therefore likely that these E proteins have similar epitope structure. Roehrig *et al.* (1983) characterized eight SLE epitopes which

overlap and form a continuum based on CBAs; these epitopes are defined by type-specific or flavivirus cross-reactive mAbs showing various biological activities. Notably, both MVE and SLE have an epitope (E-1c), defined by mAbs which have strong neutralization and HI activities and are effective in protecting mice against lethal intraperitoneal (ip) challenge with virus (Hawkes *et al.*, 1988; Mathews and Roehrig, 1984). The mAbs against the E-1c epitopes of MVE and SLE are also type-specific in ELISA reactivity to the respective virus (Roehrig *et al.*, 1983; Hawkes *et al.*, 1988).

Several sets of anti-JE mAbs have been characterized by various investigators. The unifying theme which emerges is that mutual blocking occurs between mAbs in CBAs such that clusters of overlapping epitopes are defined (Cecilia *et al.*, 1989; Kimura-Kuroda and Yasui, 1983; Kobayashi *et al.*, 1985). A set of 16 mAbs to JE has been studied by CBA and two distinct clusters of overlapping epitopes defined (Cecilia *et al.*, 1989). The remaining four mAbs are not involved in mutual blocking with these two clusters. One of the antigenic clusters consists of five epitopes which are all flavivirus cross-reactive and have HI activities. One-way inhibition, and one-way and reciprocal enhancement occur between some pairs of non-overlapping JE epitopes (Cecilia *et al.*, 1989) suggesting interaction between antigenic domains.

Epitopes on TBE are well studied with respect to function, serological specificity and spatial relationship. The most recent report for TBE (Guirakhoo *et al.*, 1989) extends the previously established epitope map of TBE (Heinz, 1986). A total of 19 epitopes on the E glycoprotein of TBE are defined by mAbs which show subtype-, type-, complex- and group-specificity in ELISA. Most of these mAbs are active in one or both the HI and neutralization assays against TBE; mAbs to two epitopes do not show either activity.

Spatial relationships between the TBE epitopes were determined by CBAs using I^{125}-labelled mAbs. As indicated by mutual blocking, 16 of the 19 TBE epitopes form

three distinct antigenic domains (A, B, C). There are three epitopes (i1, i2, i3) which do not overlap with A, B, C or each other. However, one-way inhibition occurs between non-overlapping epitopes in the same domain, and between i2, i3 and epitopes in domains B and C. Enhancement of binding occurs between i1 and epitopes in domain A; some interaction between domains is also indicated by enhancement. The antigenic domains in TBE comprise epitopes defined by mAbs of various specificities and activities. Type or subtype-specific epitopes occur in all three domains. Flavivirus group- or subgroup-reactive epitopes only occur in domain A. Epitopes in domain B are mostly complex-reactive and those in domain C are mostly subtype-specific. MAbs to domain A are more likely to have stronger HI or neutralization activities than mAbs to domains B and C.

Topological relationships betweeen epitopes of DEN-2 and YF also demonstrate more than one non-overlapping antigenic domain (Henchal *et al.*, 1985; Cammack and Gould, 1986a).

The ability of some mAbs to protect mice against lethal challenge with flaviviruses by the ip or the intracerebral (ic) route has been demonstrated for MVE, SLE, YF, DEN-2 and TBE (Brandriss *et al.*, 1986; Hawkes *et al.*, 1988; Heinz *et al.*, 1983; Henchal *et al.*, 1983; Mathews and Roehrig, 1984) indicating that the corresponding epitopes may be exploited in developing vaccination strategies. Although the most effective mAb for passive protection against ip challenge with SLE is an E-specific mAb with the highest neutralization titre (Mathews and Roehrig, 1984), neutralizing as well as non-neutralizing E-specific mAbs passively protect against lethal challenge with MVE, YF and DEN-2 (Brandriss *et al.*, 1986; Hawkes *et al.*, 1988; Henchal *et al.*, 1983). Mixtures of E-specific mAbs which were non-protective when administered singly resulted in protection against ip challenge with SLE and against ic challenge with DEN-2 (Henchal *et al.*, 1983; Mathews and Roehrig, 1984). The ability to protect against flavivirus challenge is not limited to mAbs against E, as a prM-specific mAb and

NS1-specific mAbs are also effective in passive protection against lethal challenge with DEN-2 (Henchal *et al.*, 1983; Henchal *et al.*, 1988).

1.5.3 Physical location of epitopes and a model of the TBE E protein

The physical location of six TBE neutralization epitopes is defined by single amino acids (Mandl *et al.*, 1989b): A3 (Asp 67, Ala 71), A4 (Gln 233), A5 (Glu 207), B1 (Ser 389), B4 (Tyr 384) and i2 (Lys 171). The location of epitopes in domains A, B and C is also deduced from reactivity of mAbs with discrete E proteolytic fragments. The boundary of the three TBE domains have thus been defined. Domain A consists of two separate regions (residues 50-130, 200-250); domain B consists of the region residues 301-395 and domain C is deduced to be within the sequence from residue 145 to 170 (Mandl *et al.*, 1989b).

Analysis of escape variants of YF selected with two neutralizing mAbs mapped neutralization epitopes to Asp 71 and Asn 72 in E (Lobigs *et al.*, 1987) close to epitope A3 of TBE. Bacterial expression of viral proteins has been used to map epitopes in JE and DEN-1 E protein (Mason *et al.*, 1989; Zuegel *et al.*, 1987): ten JE neutralization epitopes and two DEN-1 neutralization epitopes have been mapped to regions homologous to domain B of TBE (Met 303-Trp 396 for JE; Thr 293-Met 401 for DEN-1). Two non-neutralizing mAbs also bind to a sequence in DEN-1 E protein (Thr 76-Lys 93; Zuegel *et al.*, 1987) homologous to part of domain A of TBE.

A two-dimensional model of the TBE E protein (Fig. 1.4; Mandl *et al.*, 1989b) has been proposed, based on data from epitope mapping, topological studies, physical analysis and the disulfide linkage map for WN E (Nowak and Wengler, 1987). Physical properties of epitopes in each domain as revealed by chemical treatments (SDS, reduction and carboxymethylation, performic acid oxidation, deglycosylation, exposure to pH 5, cyanogen bromide cleavage and tryptic cleavage) are remarkably consistent

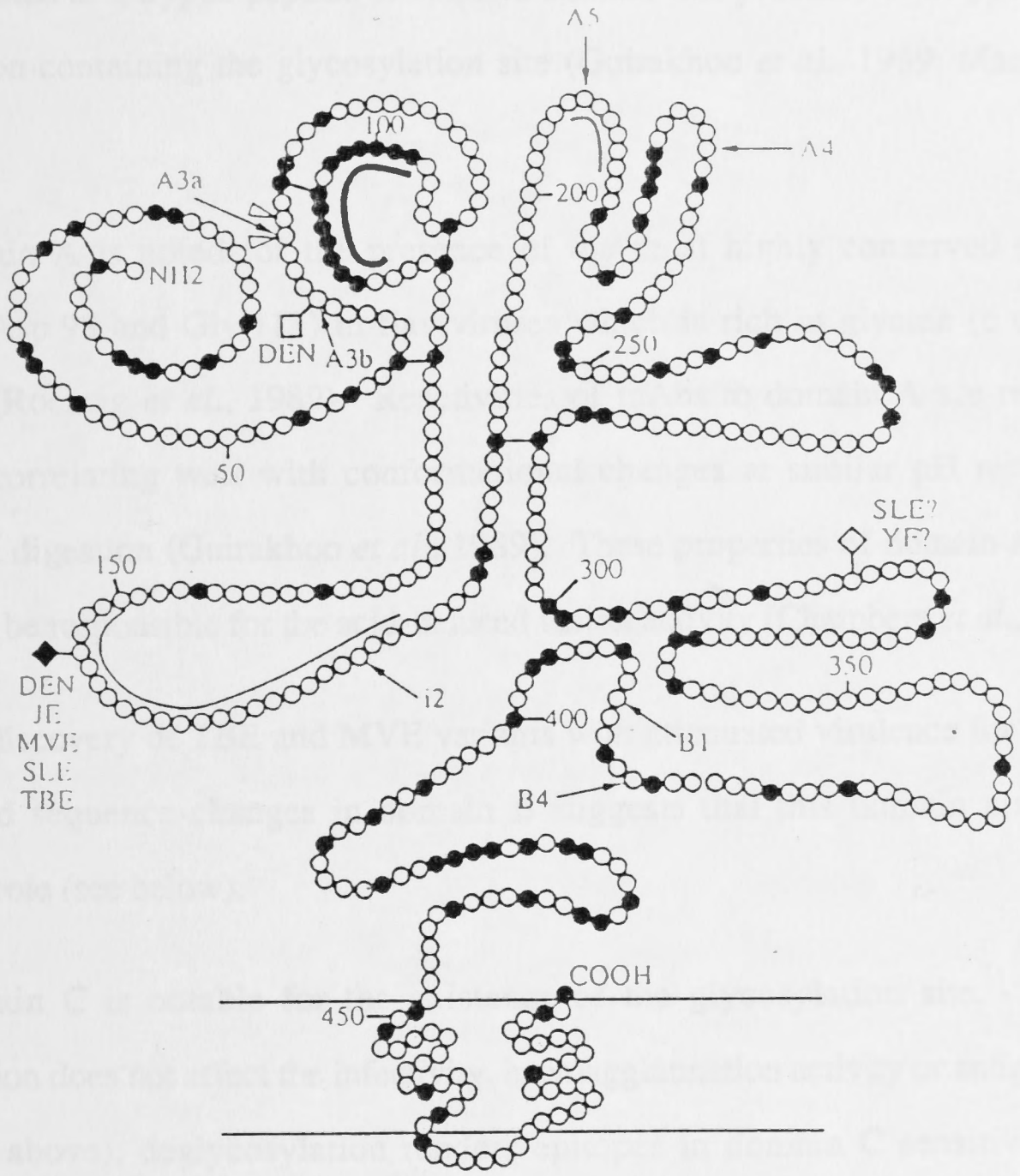


Figure 1.4 A model of TBE E protein (adapted from Mandl *et al.*, 1989b; Chambers *et al.* 1990a). Circles represent amino acids. Solid circles indicate residues absolutely conserved in the 11 flaviviruses (see Fig. 1.3) and open circles indicate nonconserved residues. Disulfide bonds are shown and diamonds indicate potential Asn-linked glycosylation sites (see text). Neutralization determinants in TBE (Heinz *et al.*, 1989b) and YF (Lobigs *et al.*, 1987) are indicated by arrows and arrowheads, respectively. Thin lines denote hyper-variable regions and the thick line a highly conserved sequence in E.

(Guirakhoo *et al.*, 1989) suggesting that domains A, B and C correspond to structurally distinct parts of E. The three domains appear to differ structurally: domain A is discontinuous and rich in disulfide bonds, domain B consists of one disulfide bond and can be isolated as a tryptic peptide of 9K, and domain C is probably in a hypervariable 'loop' region containing the glycosylation site (Guirakhoo *et al.*, 1989; Mandl *et al.*, 1989b).

Domain A is noted for the presence of the most highly conserved sequence (between Asp 98 and Gly 111) in flaviviruses which is rich in glycine (6 out of 14 residues) (Roehrig *et al.*, 1989). Reactivities of mAbs to domain A are reduced at pH 5.5-6 correlating well with conformational changes at similar pH revealed by protease K digestion (Guirakhoo *et al.*, 1989). These properties of domain A suggest that it may be responsible for the acid-induced fusion activity (Chambers *et al.*, 1990a).

The discovery of TBE and MVE variants with attenuated virulence for mice and amino acid sequence changes in domain B suggests that this domain also has an important role (see below).

Domain C is notable for the existence of the glycosylation site. Although glycosylation does not affect the infectivity, haemagglutination activity or antigenicity of TBE (see above), deglycosylation renders epitopes in domain C sensitive to SDS denaturation judging from reactivity in Western blots (Guirakhoo *et al.*, 1989). It is therefore likely that the stability of domain C is affected by glycosyaltion.

With regards to the structure and functions of E, a lot remains unresolved. A significant achievement has been the two dimensional model for the WN and TBE E proteins (Nowak and Wengler, 1987; Mandl *et al.*, 1989b). However, not enough is known about the location of epitopes in other flaviviruses to establish a unifying view of the antigenic structure. Based on sequence similarity, conservation of hydropathy character and cysteines, it is expected that the tertiary structure of E is homologous to a

large extent between flaviviruses. Thus new data on the structure of various E proteins can be compared with and used to extend the existing structural models of E. Information from epitope mapping and analysis of the three-dimensional structure of E would be of great value in predicting the regions involved in cell entry (receptor binding and fusion), neutralization and the interaction between E, prM and M during assembly of infectious virus.

1.6 Studies of virulence of flaviviruses

The flavivirus infection which occurs in humans may be visceromorphic or neuromorphic; flavivirus infection can also be less severe and involve no tissue specificity (reviewed by Monath, 1986). However, these patterns of infection are not mutually exclusive. Some flaviviruses are associated with different infections of varying severity. Viruses of the JE complex are capable of causing encephalitis in human as well as a less severe febrile illness. Similarly, infection of humans by YF can be mild, visceromorphic (hepatotropic) or neuromorphic (Monath, 1986).

Virus-associated and host-associated factors may moderate pathogenesis (see review by Brinton, 1986). Age, sex, ethnicity, genetic and immunological factors are all capable of influencing disease outcome in an infected individual. Viral factors which influence the replication and spread in the host, the ability to evade the immune response (humoral and cell-mediated immunity, interferons) and tissue tropism of virus may also determine virulence.

1.6.1 Virulence of flaviviruses for mice

The availability of a laboratory host for experimental infection of flaviviruses is essential to studies of virulence and pathogenesis. Mice are the most commonly used laboratory host for virulence studies of flaviviruses. Susceptibility of mice to flavivirus infection depends on genetic factors, age and route of inoculation (El Dadah *et al.*, 1967). Although ic inoculation of weanling mice with MVE, SLE, JE and KUN usually

results in lethal encephalitis, the outcome of ip inoculation varies greatly according to the age of the mice and the virus strain (Monath *et al.*, 1980).

Infection of mice by the ip route appears to provide a reasonable model for the pathogenesis of encephalitic flaviviruses such as MVE, JE, SLE and TBE. Viremia and neuroinvasion occur on lethal infection of mice after ip inoculation as in human encephalitis caused by flaviviruses (Albrecht, 1968). A method for assessing mouse virulence has been developed which relies on mortality ratios after ic and ip inoculation of 21-day old mice. Virulence is expressed as the ic/ip LD50 ratio (Monath *et al.*, 1980). Seven strains of MVE isolated from birds, mosquitoes or infected animals and humans from various parts of Australia and Papua New Guinea were virulent using this assay (Lobigs et al., 1988). SLE isolates from various parts of the United States fall into high, intermediate or low virulence groups by similar criteria (Monath, 1980). The highly virulent SLE strains induce high viremia in infected mice and replicate in extraneural tissues with earlier neuroinvasion. Mouse virulence shows a good correlation with clinical signs (fever, encephalitis) and histopathological markers of pathogenicity, in ic-inoculated rhesus monkeys (Monath *et al.*, 1980). There is also a correlation between mouse virulence and capacity of virus to induce viremia in the House Sparrow, a natural vertebrate host for SLE (Bowen *et al.*, 1980).

JE strains show variation in virulence for three-week old mice on the basis of differences between ic and subcutaneous LD50 values; virulence correlates with level and duration of viremia in mice and thermostability of the virus (Huang, 1957). JE isolates from humans and pigs are invariably virulent while 10% of isolates from mosquitoes have low virulence (Huang, 1982).

1.6.2 Sequence studies of variants with attenuated virulence for mice

The genetic basis for differences in mouse virulence between geographical isolates of JE and SLE (see above) is not known although differences between these JE and SLE isolates are demonstrated by RNAse T1 oligonucleotide fingerprinting (Burke *et al.*, 1985; Trent *et al.*, 1980).

Sequence analysis of laboratory derived mutants with attenuated virulence is particularly useful for assessing the contribution to virulence of particular viral proteins. Such studies have been used to compare the virulent YF Asibi strain and the avirulent 17D vaccine derivative as well as the virulent SA-14 strain of JE and its attenuated derivative (Hahn *et al.*, 1987b; Nitayaphan *et al.*, 1990). Extensive passaging of the YF Asibi strain in embryonic mouse and chick tissues gave rise to the widely used human 17D vaccine strain with markedly reduced neurotropism and viscerotropism (Theiler, 1937). Candidate vaccine strains of JE were derived by passaging in primary baby hamster kidney cells and infant mice (Huang, 1982; Yu *et al.*, 1988).

The YF Asibi strain has been sequenced completely for comparison with the 17D sequence (Rice *et al.*, 1985) in an effort to correlate attenuation of virulence with changes in the genome and protein sequence (Hahn *et al.*, 1987b). There is a 0.63% nucleotide sequence divergence between the two strains corresponding to 32 amino acid differences in the polyprotein (3430 amino acids), 12 of which are in E (493 amino acids). This biased distribution of amino acid differences indicates possible involvement of E in virulence attenuation. It has been proposed that virus binding to the cell receptor may be affected as a result of changes in the E protein, thus explaining the reduced neurotropism and viscerotropism exhibited by the vaccine strain (Hahn *et al.*, 1987b). Comparison between the JE vaccine strain SA-14-14-2 and its parent strain SA-14 gave approximately 0.4% nucleotide sequence divergence corresponding to 15 amino acid differences in the polyprotein (3432 amino acids), five of which are in E (Nitayaphan *et*

al., 1990). This biased distribution of amino acid differences is again consistent with E being involved in attenuation.

Passaging of MVE in SW13 cells and of Louping ill virus, Langat virus and three strains of WN in HeLa cells also generated variants with attenuated mouse virulence (Dunster *et al.*, 1990; Lobigs *et al.*, 1990). Comparison of the E sequences of the MVE variants implicated changes at Asp 390 as being important in attenuation, although changes in the rest of the genome may also contribute. An antigenic variant of TBE selected with neutralizing mAb was attenuated in mice and induced active immunity against lethal challenge (Heinz *et al.*, 1989); it is changed at Tyr 384 in E. This is the first demonstration for flaviviruses that a neutralization determinant may overlap with determinants of mouse virulence. An important role for the region of E close to residue 390 is also supported by the two nonconservative differences between 17D and Asibi strains of YF at 380 (Thr/Arg) and 390 (Pro/His) which correspond to Arg 388 and His 398 in MVE. These results suggest that the E proteins of flaviviruses may possess critical virulence determinants and that the region around amino acids 384 and 390 may be involved directly or indirectly in an important functional domain.

1.7 Aims and outline of thesis

The work described in this thesis aims to increase our understanding of Murray Valley encephalitis virus through focusing attention on functional domains in the envelope protein (E). There are two broad themes. In the first, covered in Chapters 2, 3 and 5, we explore the use of expression systems to isolate and characterize segments of E and to define immunologically reactive determinants in E.

In Chapter 2, we investigate the possible use of a bacterial expression system in expressing, as immunologically reactive fusion proteins, random overlapping fragments of cDNA generated by partial restriction enzyme digestion of a cDNA clone representing the 5' terminal half of the MVE genome. In chapter 3, we study the reactivity of five

neutralizing mAbs with fusion proteins generated by deletion subcloning of different segments of the MVE E gene with a view to map the corresponding epitopes in E. To further examine the epitopes in these MVE fusion proteins, they were used to generate antisera in mice for tests of activities against MVE in ELISA and neutralization assays.

It became apparent from the results obtained in Chapter 3 that only certain epitopes in the MVE E proteins could be generated in a 'native' conformation and that this would restrict us in our aims of defining functional domains using an expression system. Accordingly, in Chapter 5 we explore the possibility of using a eukaryotic expression system. Data on the yield, processing, conformation and subcellular localization of MVE structural and nonstructural proteins expressed in insect cells from cDNA cloned into a baculovirus vector are presented in Chapter 5.

The second theme of this thesis is a study of the structure-function relationships of the MVE E protein in its 'natural' environment (virion). In these studies, described in Chapter 4, we map a number of neutralization epitopes in the MVE E protein by sequencing neutralization escape variants. To determine whether these changes affect the biological properties of the virus in vivo, we examine the virulence of a number of these escape mutants for mice.

The thesis concludes with Chapter 6 in which the main threads are drawn together and the contribution of the work described in the preceeding chapters to the structure-function relationships of the MVE E protein is discussed.

CHAPTER 2

EXPRESSION OF MURRAY VALLEY ENCEPHALITIS VIRUS ANTIGENS IN *E.COLI*

2.1 INTRODUCTION

One approach to examining functional domains in the MVE E protein is to express the protein in a bacterial expression system, to screen for immunologically reactive clones with polyclonal sera against MVE and to sequence the positive clones. Reactive clones could then be subjected to a variety of further levels of analysis. Bacterial expression systems have a number of advantages in such studies. These include the ease of DNA manipulation and the possibility of obtaining large amounts of proteins which could be of value as 'reagents' for various purposes. The value of bacterial expression systems has been demonstrated for several flaviviruses. The E proteins of JE and DEN-1, and the NS1 proteins of DEN-1 and DEN-2 have been expressed in *E.coli* (Mason *et al.*, 1987b; Mason *et al.*, 1989; Zuegel *et al.*, 1987; Putnak *et al.*, 1988) and epitopes in these proteins mapped using mAbs. Fragments of the small nonstructural proteins of YF expressed in *E.coli* have been used to generate antisera which allowed the identification of NS2A, NS2B and NS4B (Chambers *et al.*, 1989). NS1 of YF expressed in *E.coli* has been used to immunize mice resulting in a reduction of neurovirulence on YF challenge (Cane and Gould, 1988). Thus in some instances the native conformation for viral proteins may be retained following expression in a bacterial system. However in others, the native conformation is not obtained; thus the JE E-ß-galactosidase fusion protein reacted with neutralizing mAbs but did not elicit neutralizing antibodies in mice (Mason *et al.*, 1989).

In this chapter we explore the application of the pEX vector system (Stanley and Luzio, 1984) to the expression of MVE proteins. The pEX expression system has been used extensively in epitope mapping for various virus systems (e.g., for *Herpesviruses* and *Coronaviruses*; see Lenstra *et al.*, 1990), but not as yet for flaviviruses. pEX plasmids have the following useful features. They are inducible (at 42°) and have a strong λ P_R promoter. Insertion in the polylinker region generates a fusion protein containing, at its N-terminus, a large portion of the lacZ (ß-galactosidase) protein. The use of a mixture of three pEX plasmids (pEX1, 2, 3) which differ in the polylinker

region, allows translation of inserts in all possible reading frames. When induced, proteins accumulate as aggregates and are partially protected from degradation (Stanley and Luzio, 1984). We aim to express certain MVE structural and nonstructural proteins (those encoded in the 5' terminal half of the genome) using this system. The strategy adopted is to clone into the pEX vector, fragments of MVE cDNA generated by partial digestion with a 4-bp recognition enzyme in order to obtain a random and overlapping set of MVE protein fragments as fusion proteins. We screen for immunologically reactive clones with hyperimmune ascitic fluid against MVE. The proteins expressed by the recombinant plasmids are then characterized with respect to size, identity, yield and stability.

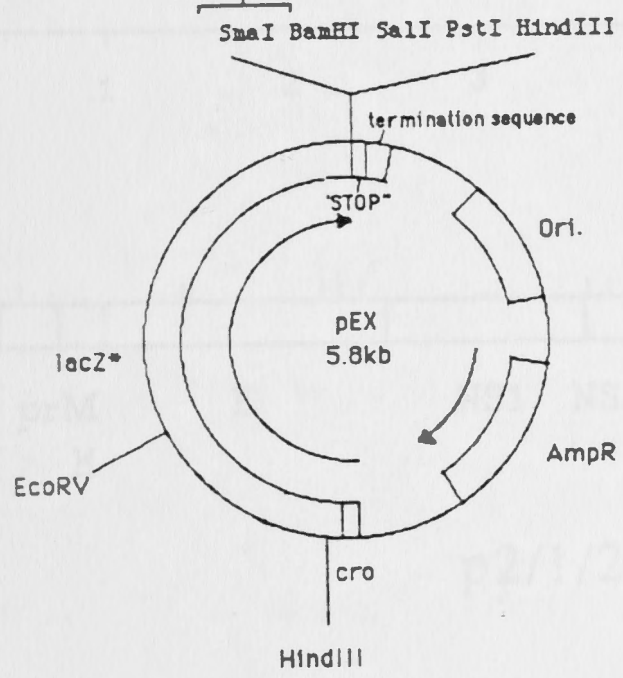
2.2 MATERIALS AND METHODS

2.2.1 Bacterial strains and plasmids

The pEX plasmids (1, 2, 3) (Fig. 2.1A; Stanley and Luzio, 1984) and the host *E.coli* MC1061/pCI857 were provided by Dr M.J. Howell (Zoology Department, Faculty of Science, Australian National University). The latter carries the plasmid pCI857 (Fig. 2.1B) encoding kanamycin resistance and the ts CI repressor gene of bacteriophage λ. Growth of bacteria was at 30° in Luria-Bertani (LB) medium (Maniatis *et al.*, 1982) supplemented with ampicillin (100μg/ml; Sigma Chemical Co. St. Louis, Missouri, USA) and kanamycin (40μg/ml; Sigma).

Plasmid p2/1/22 (Fig. 2.2), provided by Dr L. Dalgarno (Biochemistry Department, Faculty of Science, Australian National University), is from an MVE genomic library (Rice *et al.*, 1988). It comprises the 1.9 kb plasmid vector pMT21 (from Dr H.V. Huang, Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri, USA) which codes for ampicillin resistance, and the 5' terminal 5.4 kb of the MVE genome coding for C, prM, E, NS1, NS2A, NS2B and half of NS3; the complete sequence of p2/1/22 is established (Dalgarno *et al.*, 1986).

A. pEX1 GAATTAATTCCCGGG
pEX2 G AATTCCCGGG
pEX3 G CCCGGG



B.

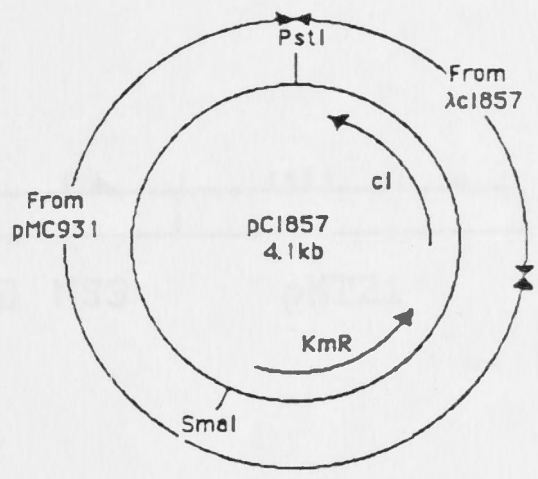


Figure 2.1 (A) pEX plasmids 1, 2 and 3. The three pEX plasmids are represented diagrammatically showing the gene for ampicillin resistance (Amp^R), the *cro* gene, the lacZ* gene (missing ≈60 bp and 55 bp at the 5' and 3' termini of lacZ, respectively), and the pBR322 origin of replication (Ori). Arrows indicate the direction of transcription and length of genes. Each plasmid also contains a polylinker, stop codons in three reading frames ('STOP') and a fragment from phage fd containing a transcription termination sequence. The three plasmids differ only in the polylinker region; pEX1 is eight and four base pairs longer than pEX3 and pEX2, respectively. Data are adapted from Stanley and Luzio (1984).

(B) Plasmid pCI857. pCI857 (4.1 kb) is made up of the pMC931 plasmid, the kanamycin resistance gene and the ts CI repressor gene from phage λ.

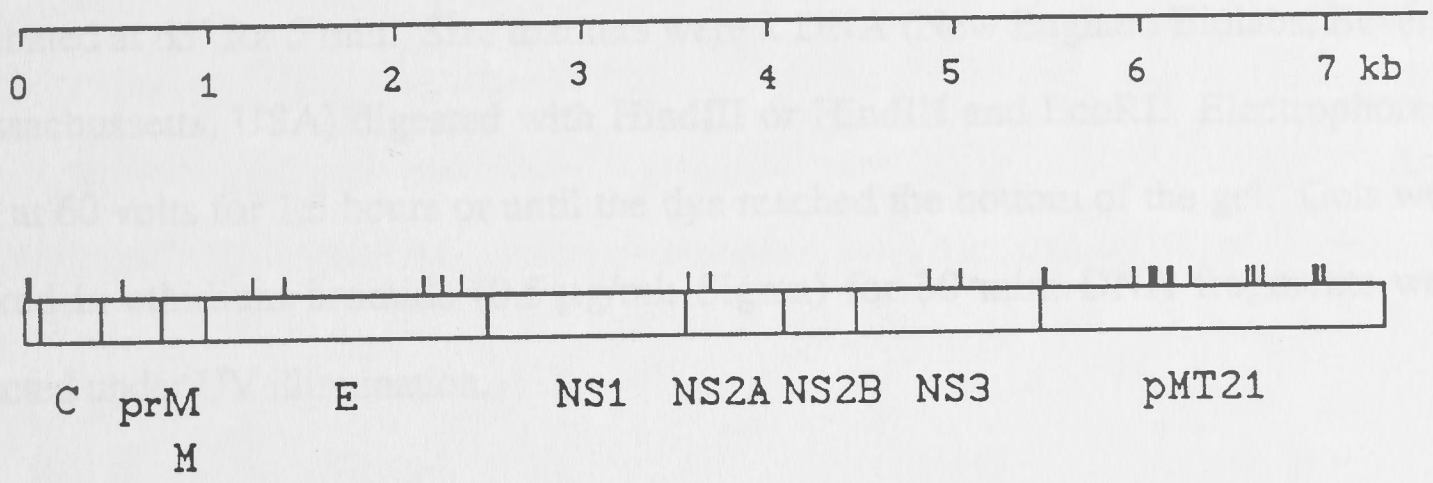


Figure 2.2 Sau3A restriction map of linearized p2/1/22. Locations of Sau3A restriction sites were determined by a computer search of the pMT21 sequence (H. V. Huang, personal communication) and the MVE genome sequence (Dalgarno *et al.*, 1986), and are shown as vertical lines. The p2/1/22 is linearized at the 5' terminus of the MVE insert. It contains the 5' untranslated region (≈90 bp), coding regions for C, prM, E, NS1, NS2A, NS2B and NS3 proteins and the pMT21 plasmid (1895 bp).

2.2.2 Agarose gel electrophoresis

Separation of DNA fragments was on 1% agarose. Agarose was prepared by dissolving agarose (1% w/v; Ultra-pure DNA grade, Bio-Rad Laboratories, Richmond, California, USA) in TAE buffer (40 mM Tris-HCl, pH 7.8, 5 mM sodium acetate, 1 mM EDTA). A horizontal slab gel (≈12 ml; 50 mm x 75 mm) was used. One tenth of a volume of loading buffer containing 15% Ficoll type 400 (Pharmacia Fine Chemicals, Uppsala, Sweden), 0.1 M EDTA, 20 mM Tris-HCl (pH 7.8), 2.5 mM sodium acetate and 0.25% (w/v) Orange G was added to the sample and the mixture incubated at 65° for 5 min. Size markers were λ DNA (New England Biolabs, Beverly, Massachussetts, USA) digested with HindIII or HindIII and EcoRI. Electrophoresis was at 60 volts for 1.5 hours or until the dye reached the bottom of the gel. Gels were soaked in ethidium bromide (0.5 μg/ml; Sigma) for 30 min; DNA fragments were detected under UV illumination.

2.2.3 DNA manipulations

Plasmid DNA for cloning, restriction analysis and sequencing was extracted by alkaline lysis (Maniatis *et al.*, 1982). Bacteria in LB medium containing antibiotics were incubated overnight at 30°. Cell pellets from 3 ml of culture were collected in Eppendorf tubes by centrifugation for 2 min and resuspended in 100 μl of ice-cold 50 mM glucose (Sigma), 10 mM EDTA, 25 mM Tris-HCl (pH 8.0) and 4 mg/ml lysozyme (P-L Biochemicals, Milwaukee, Wisconsin, USA). After 5 min at room temperature, 200 μl of ice-cold 0.2 M NaOH, 1% SDS was added, and the mixture incubated on ice for 5 min. Potassium acetate solution (150 μl; Maniatis *et al.*, 1982) was then added, mixed and incubated for 5 min. The mixture was centrifuged for 5 min (Eppendorf) at 4°. The supernatant was extracted with redistilled phenol (British Drug Houses, Port Fairy, Australia): isoamylalcohol (Ajax Chemicals, Sydney, Australia): chloroform (Ajax) (25:1:24) twice. DNA was precipitated with ethanol (2 volumes), recovered by centrifugation and resuspended in 5 mM TE buffer (5 mM Tris-HCl, pH 8, 0.5 mM EDTA) containing DNAase-free pancreatic RNAase A (20 μg/ml;

Boehringer-Mannheim, Mannheim, West Germany). Digestion was at 37° for 30 min.

For restriction enzyme (RE) digestion, plasmid DNA was incubated with enzymes (New England Biolabs; 2 units per μg DNA) at 37° for 1 hour unless stated otherwise. RE buffers were 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin (Bio-Rad) and NaCl at 150 mM, 100 mM, 50 mM or 0 mM according the manufacturer's recommendation. SmaI digestion was in 10 mM KCl, 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$ and 0.1 mg/ml bovine serum albumin at 25°. Incubation with SmaI was carried out first when other enzymes were also used. Incubation with other enzymes then followed in appropriate buffer conditions without removing the KCl. Enzyme digestion was stopped by incubation at 70° for 10 min.

2.2.4 Construction of pEX-MVE recombinant plasmids

For the partial digestion of p2/1/22, DNA (2 μg) was incubated with Sau3A (0.2 units) at 37° for 10 min, heated at 70° for 10 min and used directly for cloning without size selection. pEX vector DNA was prepared for ligation with Sau3A-digested cDNA by digesting (37°, 1 hour) a mixture of pEX1, 2 and 3 (1.5 μg each) with BamH1 (10 units). Calf intestinal alkaline phosphatase (2 units; Boehringer) was added and the mixture incubated at 37° for 30 min and heated at 70° for 10 min. DNA was twice extracted with phenol/chloroform and precipitated with sodium acetate (0.3 M, pH 5.2) and ethanol (2 volumes).

For ligation, vector DNA (0.4 μg), Sau3A-digested p2/1/22 (≈15 ng) and T4 DNA ligase (100 units, New England Biolabs) were incubated in a final volume of 10 μl containing 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 20 mM dithiothreitol (DTT; Boehringer), 1 mM ATP and 50 μg/ml bovine serum albumin. The precise molar ratio of vector to insert DNA could not be calculated. Incubation was at 14° for 16 hours followed by heat inactivation at 70° for 10 min. Dephosphorylated vector

DNA was ligated alone as control to determine levels of uncut and re-ligated vector DNA.

2.2.5 Preparation of competent *Ecoli* and transformation (C. M. Rice, personal communication)

E.coli MC1061/pCI857 cells were harvested from 50 ml log phase culture (OD 630nm = 0.6) by centrifugation at 8000 rpm for 10 min, resuspended in 5 ml of ice-cold 0.1 M $CaCl_2$, held on ice for 20 min and centrifuged again. Cell pellets were resuspended in 0.5 ml of 0.1 M $CaCl_2$ plus 15% glycerol (May and Baker, Sydney, Australia) and stored in liquid nitrogen in 50 µl aliquots. For transformation, competent cells were diluted in 18 volumes of 0.1 M $CaCl_2$ and divided into two equal portions. Ligated DNA mixtures (10 µl) were added to each and the cells were held on ice for 30 min followed by a heat shock at 43° for 45 seconds. LB broth (30°) was then added to dilute the mixtures 10-fold. Cells were incubated for 1.5 hours at 30° and recovered by centrifugation at 4000 rpm for 10 min, resuspended, spread and incubated at 30° on LB plates containing 1.5% (w/v) Bacto-agar (Difco Laboratories, Detroit, Michigan, USA), ampicillin and kanamycin.

2.2.6 Hyperimmune ascitic fluid (HIAF)

Anti-MVE HIAF was provided by Dr R. C. Weir (Biochemistry Department, Faculty of Science, Australian National University). UV-inactivated MVE-1-51 (the prototype strain; French, 1952) was used to inoculate animals, the animals were boosted with live virus and ascitic fluids harvested (Tikasingh *et al.*, 1966). A single batch of HIAF was used for the work described in this Chapter. Its ELISA titre was 128,000. This antibody preparation immunoprecipitated MVE C, prM, M and E as well as NS1, NS3 and NS5 (Lee *et al.*, 1990; Lobigs *et al.*, 1986).

2.2.7 Immunological screening

Colonies expressing MVE antigens were identified by colony blots following Stanley (1983) and M. J. Howell (personal communication). For initial screening,

colonies (≈2000) which grew on LB plates containing 100 µg/ml ampicillin and 40 µg/ml kanamycin were tested. For subsequent confirmation of positive clones and for estimation of percentage of immunoreactive colonies, approximately 100 colonies were tooth-picked to LB plates. After incubation at 30° for 16 hours, the colonies were transferred to sterile, dry nitrocellulose (0.45 µm, Schleicher and Schuell, Dassel, W. Germany) by blotting. Membranes were then placed on two sheets of 3MM paper (Whatman Ltd.,, Kent, England) soaked in LB medium containing antibiotics. Expression of fusion proteins containing a hybrid protein *cro* -ß-galactosidase, termed ß-gal*, was induced by incubation for 2 hours at 42°. Membranes were then placed, colony-side up, on 3 sheets of 3MM paper moistened with 5% sodium dodecyl sulphate (SDS; Bio-Rad), and baked at 100° for 15 min. SDS was removed by soaking membranes for 5 min (x4) in wash buffer (WB) which contained 0.05% Tween-20 (Sigma) in phosphate-buffered saline (PBS; 0.15 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.5). The membranes were then treated as follows: (1) 30 min in WB containing 5% (w/v) non-fat milk powder (Diploma, Melbourne, Australia); (2) 30 min in WB containing 0.1 mg/ml DNAase (bovine pancreatic; Sigma); (3) 3 x 5 min in WB; (4) 60 min in WB containing anti-MVE HIAF (1/400); (5) 3 x 5 min in WB; (6) 60 min in WB containing horseradish peroxidase coupled to goat anti-(mouse IgG) (1/1500; Bio-Rad); (7) 3 x 5 min in WB and 5 min in PBS. Colour devolpment was for 30-60 min at room temperature in Tris-buffered saline (0.2 M NaCl, 0.05 M Tris-HCl, pH 7.4) containing 4-chloro-1-naphthol (0.5 mg/ml; Bio-Rad) and hydrogen peroxide (0.02% ; v/v; Ajax).

2.2.8 DNA sequence analysis

Recombinant plasmid DNA (1 µg) was incubated with HindIII (5 units) at 37° for 1 hour followed by 10 min at 70°. The 3' recessed ends were labelled by addition of Klenow fragment (5 units; New England Biolabs), α-^{32}P-dCTP (20 µCi; Amersham International Ltd., Amersham, U.K.), an excess (0.5 mM) of the other three dNTPs and ß-mercaptoethanol (ß-ME; Sigma) to a final concentration of 1 mM. Incubation

was at 37° for 30 min and then 70° for 10 min. EcoRV (see Fig. 2.1) was then added (5 units) and the mixture incubated (37°, 1 hour) to produce fragments labelled at the 3' ends. These were separated on 8% (w/v) preparative acrylamide gels (Bio-Rad; 40 cm long, 0.4 mm thick) cross-linked with N,N'-bisacrylylcystamine (BAC; Bio-Rad) (19:1). Electrophoresis was at 200 V for 16 hours in TBE buffer (0.1 M Tris-HCl, pH 8.3, 0.1 M boric acid, 2 mM EDTA). Size standards were λ DNA (0.2 μg) digested with HindIII and labelled with α-^{32}P-dCTP as above. The gel was exposed for 15 min to Fuji RX-100 film for autoradiography. DNA fragments were localized, excised and extracted by incubation of gel slices with ß-ME and then with DE-52 DEAE-cellulose resin (Whatman) (Garfinkel *et al.*, 1983). Fragments were subjected to cleavage reactions (Maxam and Gilbert, 1980) with hydrazin (Eastman Kodak Co., Rochester, New York, USA), dimethyl sulphate (Aldrich Chemical Co., Milwaukee, Wisconsin, USA), formic acid, sodium hydroxide and piperidine (Fisher Scientific Co., Fair Lawn, New Jersey, USA) for sequence analysis as described by Sambrook *et al.* (1989). Cleavage products were separated on sequencing gels (80 cm long and 0.4 mm thick) containing 5% acrylamide: bisacrylamide (19:1). They were dried under vacuum at 80° in a slab-gel dryer (Bio-Rad) and exposed to Fuji RX-100 films for autoradiography. Intensifying screens (Kodak) were sometimes used.

2.2.9 Induction of MVE fusion proteins and analysis of bacterial lysates by polyacrylamide gel electrophoresis

Bacterial colonies selected by immunological screening were grown in LB medium containing ampicillin (100 μg/ml) and kanamycin (40 μg/ml). Incubation was in an orbital shaker at 30° overnight. The overnight-cultures were diluted to an absorbance (630 nm) of 0.05 with LB medium containing antibiotics and incubated for a further 1 hour as above. The temperature was raised to 42° for induction of fusion protein synthesis. Bacteria (1.5 ml) were collected after 2 hours by centrifugation for 2 min (Eppendorf), resuspended to 75 μl in SDS-polyacrylamide gel electrophoresis (PAGE)

sample buffer (Laemmli, 1970) and heated for 2 min at 100°. Lysates were sonicated (20 seconds), centrifuged (2 min) to remove debris and stored at -20°.

Lysates (1 µl) were diluted in SDS-PAGE sample buffer containing 5% (v/v) ß-ME, heated (2 min, 100°) and electrophoresed on vertical slab gels (70 mm x 100 mm x 0.75 mm) (Laemmli, 1970). The stacking and resolving gels contained 3 and 7.5% acrylamide respectively. Gels were stained for 30-60 min in 0.25% (w/v) Coomassie Brilliant Blue R-250 (Serva, Heidelberg) in 10% (v/v) acetic acid-45% (v/v) methanol (Ajax). Destaining was in 23% (v/v) ethanol-10% (v/v) acetic acid. Mol. wt. standards (43-200K; Bio-Rad) were electrophoresed and stained on the same gel.

2.3 RESULTS

2.3.1 Construction of MVE-pEX recombinant plasmids

The initial objective was to prepare a library containing genes for the MVE proteins prM, E and NS1 in the pEX vector. Plasmid p2/1/22, which derives from ligation of the pMT21 vector (see Section 2.2.1) with cDNA coding for the 5' terminal half of the MVE genome, was the source of MVE sequences. Sau3A digestion was used to generate a range of fragments for cloning. Sau3A was chosen for three reasons. First, as a 4-bp restriction enzyme, it cleaves frequently and hence a partial digest will, in principle, generate random overlapping fragments. Second, it generates fragments with BamHI compatible ends. Finally, the number of Sau3A sites in the MVE E gene was greater than that for other 4-bp restriction enzymes examined. There are 27 predicted Sau3A sites in p2/1/22, including 12 in the insert and 15 in the vector (Fig. 2.2). There are five Sau3A sites in the E gene and thus a variety of E gene fragments could be generated. It was noted that there is no Sau3A site in the C gene and thus Sau3A fragments coding for C would contain the 5' untranslated region (Fig. 2.2). The expression of C from these fragments was expected to be disrupted by in-frame nonsense codons present in this region.

To obtain overlapping restriction fragments, a partial digestion of 2 µg of p2/1/22 DNA with Sau3A was performed. Fragments in the restriction digest ranged from below 100 bp to around 2 kb (Fig. 2.3). As the largest completely digested fragment was expected to be ≈1.3 kb, this indicated that partial digestion had occurred. To minimize the loss of small fragments (≈100 bp or less) from the E gene (Fig. 2.2), the partial digest was ligated, without extraction, to a mixture of BamHI-digested, dephosphorylated pEX1, 2 and 3 vectors. The mixture was used to transform *E.coli* MC1061/pCI857. From 0.1 µg of total DNA, approximately 10,000 ampicillin and kanamycin resistant colonies were obtained.

2.3.2 Immunoscreening of bacterial colonies

Anti-MVE HIAF was used to identify colonies expressing recombinant MVE proteins. Colonies were grown on LB plates containing antibiotics overnight and transferred to nitrocellulose. Adherent cells were incubated at 42° for 2 hours to induce synthesis of the ß-gal* fusion protein, and were lysed by SDS treatment at 100°. MVE-specific proteins were detected using anti-MVE HIAF and anti-(mouse IgG) coupled to peroxidase. Approximately 1% of the colonies reacted with anti-MVE HIAF. The low frequency was not surprising because of the presence of pMT21 sequences during cloning, the possibility of out-of-frame translation of MVE sequences and of insertion of MVE sequences in the reverse orientation. Eleven immunoreactive clones were eventually isolated. These were designated 88-I, 2-II, 4-II, 48-II, 63-II, 60-V, 81-V, 92-V, 103-V, 9-VI and 11-VI. One of the eleven immunoreactive clones (48-II) was unstable on propagation: the recombinant plasmid (p48-II) was replaced by pEX plasmids resulting in a loss of immunoreactivity.

2.3.3 Size of inserts encoding immunoreactive peptides

As a first step in identifying the positions in the MVE genome represented by the immunoreactive fusion proteins, the sizes of the inserts in the recombinant pEX plasmids were determined. Using alkaline lysis (Materials and Methods), plasmid DNA

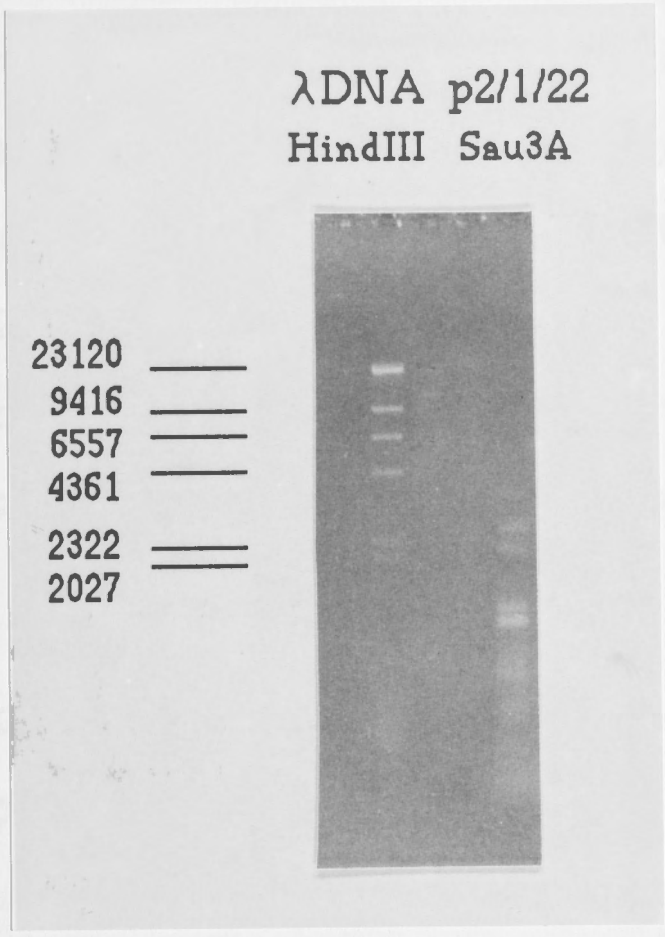


Figure 2.3 Agarose gel electrophoresis of a Sau3A-partial digest of p2/1/22. p2/1/22 (2 µg) was incubated with Sau3A (0.2 units) in RE buffer containing 0.15 M NaCl at 37° for 10 min and electrophoresed on 1% agarose gel.

was extracted from the 11 positive clones for RE digestion with SalI and SmaI; these sites flank the BamHI site in the polylinker (Fig. 2.1). The size of the insert was determined after digestion with these enzymes. Plasmids p63-II, p9-VI, p81-V and p48-II each released one fragment (0.4, 0.9, 1.5 and 2.8 kb, respectively) corresponding to the insert, together with the vector (5.8 kb) and pCI857 (4.1 kb) (results not shown). This indicated that there were probably no SmaI or SalI sites in these inserts. Plasmids p88-I, p2-II, p4-II, p60-V, p92-V, p103-V and p11-VI released fragments smaller than 200 bp which could not be accurately sized. It was not possible to identify inserts from their sizes because p2/1/22 contains several small Sau3A fragments of similar size and multiple inserts may be present in some recombinant plasmids.

2.3.4 Characterization and mapping inserts encoding immunoreactive peptides

To map the inserts in the 11 clones, two approaches were used: restriction enzyme analysis followed by DNA sequence analysis.

The large inserts in p9-VI, p81-V and p48-II were characterized by digesting with MluI and PstI enzymes and agarose gel electrophoresis (results not shown); a Sau3A fragment from the E gene (corresponding to nucleotides 1403-2149; 0.75 kb) was identified as present in p9-VI and a Sau3A fragment from the prM-E gene (nucleotides 534-2149; 1.6 kb) as present in p48-II (Fig. 2.4). However, since inserts in p9-VI and p48-II, 0.9 and 2.8 kb respectively, were larger than these two Sau3A fragments in each instance, it was concluded that p9-VI and p48-II also contained additional Sau3A fragments. Plasmid p81-V did not contain the above fragments from similar analysis (results not shown).

Sequence analysis was performed on all clones. The eleven plasmids were digested with HindIII and end-labelled using α-^{32}P-dCTP. A second digestion with EcoRV allowed isolation of fragments labelled solely at the one of the 3' ends.

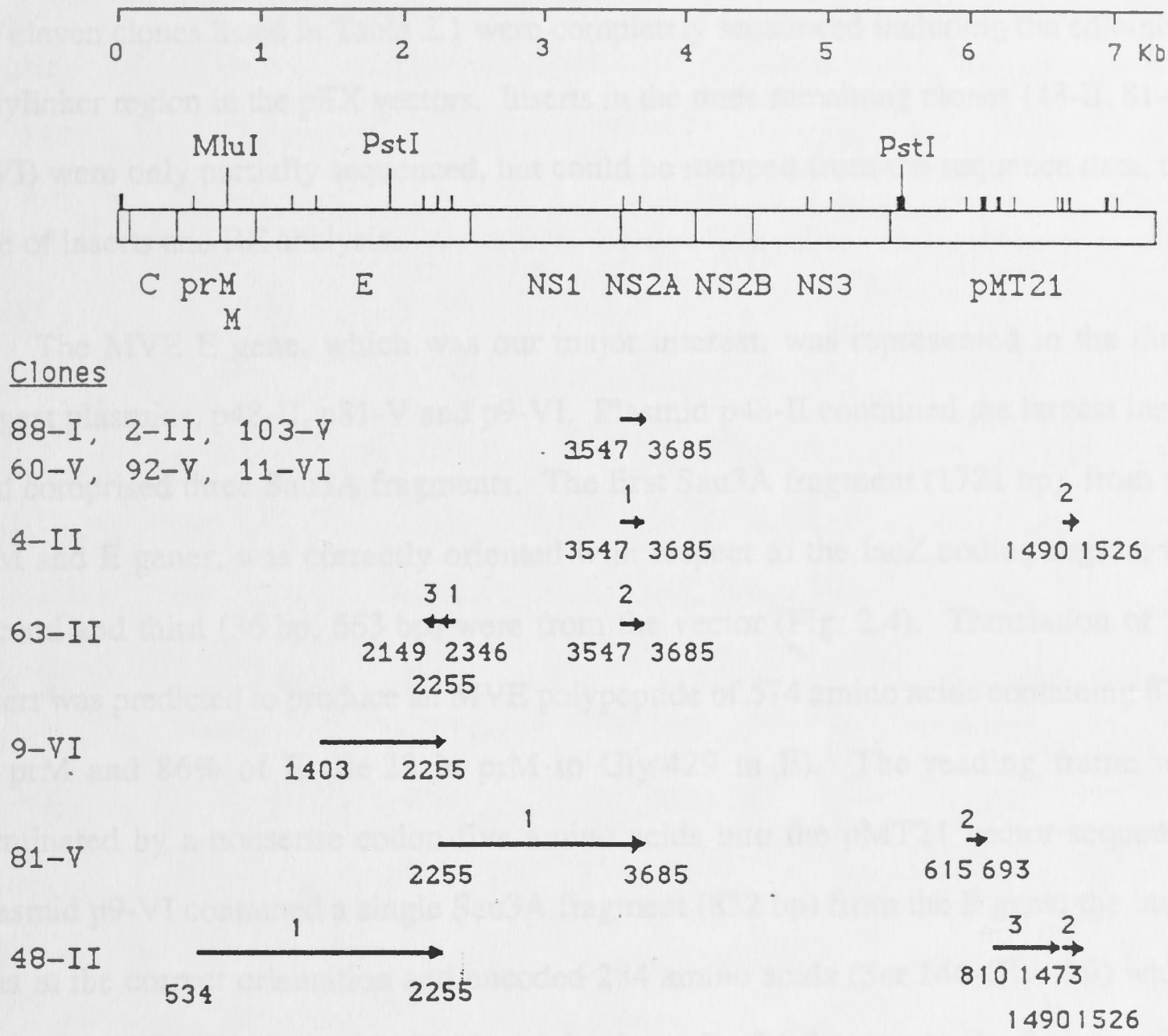


Figure 2.4 Orientations and sizes of inserts in recombinant plasmids. The locations of Sau3A fragments are indicated by alignment with the MVE genome and with the pMT21 sequence. MVE fragments are numbered from the 5' terminal nucleotide of MVE-1-51 RNA (Dalgarno *et al.*, 1986). pMT21 fragments are numbered from the 5' terminal nucleotide in the sequence linearized at the EcoRI site at the end of the polylinker (H. V. Huang, personal communication). Arrows indicate the orientation of these fragments in the recombinant plasmids: (→), orientation identical to lacZ* coding region; (←), opposite orientation. Numbers above the fragments indicate their relative positions in the insert.

Sequencing reactions were performed on these fragments (Maxam and Gilbert, 1980). Up to 400 bases from the 3' termini of the inserts were sequenced. Inserts in eight of the eleven clones listed in Table 2.1 were completely sequenced including the adjoining polylinker region in the pEX vectors. Inserts in the three remaining clones (48-II, 81-V, 9-VI) were only partially sequenced, but could be mapped from the sequence data, the size of inserts and RE analysis.

The MVE E gene, which was our major interest, was represented in the three largest plasmids, p48-II, p81-V and p9-VI. Plasmid p48-II contained the largest insert and comprised three Sau3A fragments. The first Sau3A fragment (1721 bp), from the prM and E genes, was correctly oriented with respect to the lacZ coding region; the second and third (36 bp, 663 bp) were from the vector (Fig. 2.4). Translation of the insert was predicted to produce an MVE polypeptide of 574 amino acids containing 87% of prM and 86% of E (Ile 23 in prM to Gly 429 in E). The reading frame was terminated by a nonsense codon five amino acids into the pMT21 vector sequence. Plasmid p9-VI contained a single Sau3A fragment (852 bp) from the E gene; the insert was in the correct orientation and encoded 284 amino acids (Ser 146-Gly 429) which represent 57% of E. Plasmid p81-V contained two Sau3A fragments, the first spanning the E, NS1 and NS2A genes (1430 bp) in the correct orientation while the second (78 bp) was from the vector. Translation of the insert was predicted to produce an MVE polypeptide of 477 amino acids containing the C-terminus of E (72 amino acids), all of NS1 (352 amino acids) and part of NS2A. This protein therefore comprised Ser 430 in E to Asp 53 in NS2A. Translation was terminated by a nonsense codon seven amino acids into the pMT21 sequence.

Plasmids p88-I, p2-II, p103-V, p60-V, p92-V, p11-VI, p4-II and p63-V were sequenced completely through the inserts and the 5' adjoining polylinker region. From this it was established that p88-I, p2-II and p103-V were recombinants of pEX1

TABLE 2.1

Properties of immunoreactive clones

Clone	Immuno-reactivity[a]	Size of insert[b]	Size of fusion protein[c]	Yield[d]	Gene position[e]
88-I	++	<200bp	125K (127K)	+	NS2A
2-II	+	<200bp	125K (127K)	+	NS2A
103-V	+	<200bp	125K (127K)	+	NS2A
60-V	+++	<200bp	125K (127K)	+++	<u>NS2A</u>
92-V	++	<200bp	125K (127K)	+	<u>NS2A</u>
11-VI	+++	<200bp	125K (127K)	+++	<u>NS2A</u>
4-II	+++	<200bp	125K (127K)	+++	<u>NS2A</u>
63-II	++	≈400bp	134K (134K)	+	NS2A
9-VI	+++	≈900bp	158K (153K)	++	E
81-V	+++	1.5K	174K (175K)	+	E, NS1, NS2A
48-II	+	2.8K	not detected (184K)	not detected	prM, E

[a] Immunoreactivity: +, weak; ++, moderate; +++, strong.

[b] Sizes of inserts were estimated by agarose gel electrophoresis (see text).

[c] Size of fusion proteins were estimated by SDS-PAGE of lysates prepared from induced bacterial cultures (see Fig. 2.6). Size of fusion proteins in brackets was predicted based on the size of ß-gal* (122K) and the amino acid composition of the MVE peptides (See text).

[d] Yields of fusion proteins were estimated by comparison with the yield of ß-gal* (50 µg/ml): +, 5-10 µg/ml; ++, 10-30 µg/ml; +++, 30-40 µg/ml.

[e] MVE protein genes in each clone. Gene fragments in the reverse orientation were not listed and fragments inserted in an incorrect reading frame were underlined.

plasmid, that p63-II was a recombinant of pEX2 and that p60-V, p92-V, p11-V and p4-II were recombinants of pEX3.

Plasmids p88-I, p2-II and p103-V contained an identical 138 bp NS2A gene insert in the correct orientation (Fig. 2.4). The inserts were predicted to be translated as Asp 7-Asp 53 of NS2A (Fig. 2.5A).

Plasmid p63-II contained three Sau3A fragments in the insert (Fig. 2.4). The first (91 bp) and third (106 bp) were from the E gene. However, the orientation of both fragments was reversed. The second fragment (138 bp) was from the NS2A gene. The orientation of this fragment was correct. Translation of this insert was predicted to produce a peptide of 110 amino acids, including 47 amino acids of NS2A (Asp 7-Asp 53) (Figure 2.5B).

Plasmids p60-V, p92-V and p11-VI also contained the 138 bp NS2A insert, but the vector was pEX3 in each case (Fig. 2.5C). Because the insertion was out-of-frame, translation was expected to produce a peptide of 45 amino acids in a different reading frame (Fig. 2.5C). This is inconsistent with the strong immunoreactivity observed for these clones (see below).

Plasmid p4-II contained two Sau3A fragments (Fig. 2.4). The first was the correctly oriented 138 bp fragment from the NS2A gene and the second (36 bp) was from pMT21 (Fig. 2.4). As the vector was pEX3 the predicted translation product was an identical fusion protein to that in 60-V, 92-V and 11-VI (Fig. 2.5D).

Each of the immunoreactive clones contained at least one correctly oriented fragment from MVE cDNA, consistent with the immunoblotting data. The question of whether the expected reading frames were used for translation is addressed in the following sections. All 11 recombinant plasmids were derived from Sau3A fragments and three of them (48-II, 81-V, 9-VI) contained internal Sau3A sites indicating incomplete digestion (Fig. 2.4), consistent with the cloning procedures used to generate

Figure 2.5 Nucleotide and deduced amino acid sequences of inserts in recombinant plasmids. The nucleotide sequences of p88-I, p2-II, p103-V, p60-V, p92-V, p11-VI, p4-II and p63-II were determined (Maxam and Gilbert, 1980) and presented from the 3' end of the lacZ* coding region (12 bp from the polylinker) to the 3' end of the insert. The amino acid sequence encoded in these inserts was predicted by extending the reading frame for ß-galactosidase. Asterisks indicate termination codons, after which amino acid sequence was not shown. The Sau3A sites which flank each MVE or pMT21 fragment in the insert are indicated. The NS2A peptide Asp7-Asp53 is underlined. Sequences of inserts in p4-II and p63-II are abbreviated, the NS2A gene fragment in these inserts is identical to the insert in (A).

A. 88-I, 2-II, 103-V

Polylinker

lacZ* pEX1 MVE NS2A

```
TCA GTA TCG GCG GAA TTA ATT CCC GGG GAT CCT TTT CAG TTA GGC CTT CTG GTG
ser val ser ala glu leu ile pro gly asp pro phe gln leu gly leu leu val

ATG TTT CTG GCC ACC CAG GAG GTC TTG AGG AAG AAG TGG ACG GCC AGA CTT ACT
met phe leu ala thr gln glu val leu arg lys lys trp thr ala arg leu thr

CTG CCA GCA GCG GTT GGG GCT CTG CTA GTC CTC CTC CTT GGG GGC ATT ACC TAC
leu pro ala ala val gly ala leu leu val leu leu leu gly gly ile thr tyr

ACT GAT C
thr asp
```

B. 63-II

Polylinker

lacZ* pEX2 MVE E

```
TCA GTA TCG GCG GAA TTC CCG GGG ATC CAT GAC ATT CCT....CCT ACT GAT CCT
ser val ser ala glu phe pro gly ile his asp ile pro....pro thr asp pro
```

MVE NS2A MVE E

```
TTT....TAC ACT GAT CCA AAG TCC....TGG TGA TTG ATC
phe....tyr thr asp pro lys ser....trp  *
```

C. 60-V, 92-V, 11-VI

Polylinker

lacZ* pEX3 MVE NS2A

```
TCA GTA TCG GCG GCC CGG GGA TCC TTT TCA GTT AGG CCT TCT GGT GAT GTT TCT
ser val ser ala ala arg gly ser phe ser val arg pro ser gly asp val ser

GGC CAC CCA GGA GGT CTT GAG GAA GAA GTG GAC GGC CAG ACT TAC TCT GCC AGC
gly his pro gly gly leu glu glu glu val asp gly gln thr tyr ser ala ser

AGC GGT TGG GGC TCT GCT AGT CCT CCT CCT TGG GGG CAT TAC CTA CAC TGA TC
ser gly trp gly ser ala ser pro pro pro trp gly his tyr leu his  *
```

D. 4-II

Polylinker

lacZ* pEX3 MVE NS2A

```
TCA GTA TCG GCG GCC CGG GGA TCC TTT TCA GTT AGG CCT TCT....CTA CAC TGA
ser val ser ala ala arg gly ser phe ser val arg pro ser....leu his  *
```

pMT21

```
TCC AGT TCG ATG TAA CCC ACT CGT GCA CCC AAC TGA TC
```

the library. The presence of more than one Sau3A fragment in four plasmids indicated that the molar ratio of vector to insert during ligation was not high enough to ensure incorporation of a single Sau3A fragment in each recombinant plasmid.

2.3.5 Yields of MVE fusion proteins

The yields of MVE fusion proteins from *E.coli* were roughly quantitated. The eleven clones were grown in culture and induced for fusion protein synthesis. Extracts containing equivalent amounts of bacterial cells were analysed by SDS-PAGE, stained with Coomassie Blue and compared with protein standards. The uninduced culture of pEX transformants contained trace amounts of the truncated ß-galactosidase protein, denoted ß-gal*; induction resulted in a yield of approximately 50 µg of ß-gal* per ml of culture (results not shown). Yields of fusion proteins were from 5 to 40 µg/ml (Table 2.1). The lowest was for 81-V and 63-II while the highest was for 4-II, 11-VI and 60-V; the latter three colonies were also among the most immunoreactive (Table 2.1). Colonies containing large inserts gave poor yields in general. No fusion protein was detected for the unstable 48-II although ß-gal* was detected (see Discussion). Of the three clones producing MVE E fusion proteins, only 9-VI gave a good yield (≈30 µg/ml).

2.3.6 Molecular weights of MVE fusion proteins

Molecular weights (mol. wts.). of fusion proteins were predicted from sequencing and mapping data (see above). Experimental mol. wts. were determined by SDS-PAGE. The nominal mol. wt. of ß-gal* was 122K (Fig. 2.6). The experimental mol. wts. of fusion proteins from ten clones was ≈125-174K (Table 2.1), corresponding to ≈3-52K added to the C-terminus of ß-gal*.

The mol. wts. of the fusion proteins in clones 81-V and 9-VI (174K and 158K) was consistent with the fusion of 477 and 284 amino acids respectively with ß-gal*, indicating that translation of the MVE coding regions in the appropriate reading frame had occurred. Clone 81-V was predicted to encode E-NS1-NS2A which would

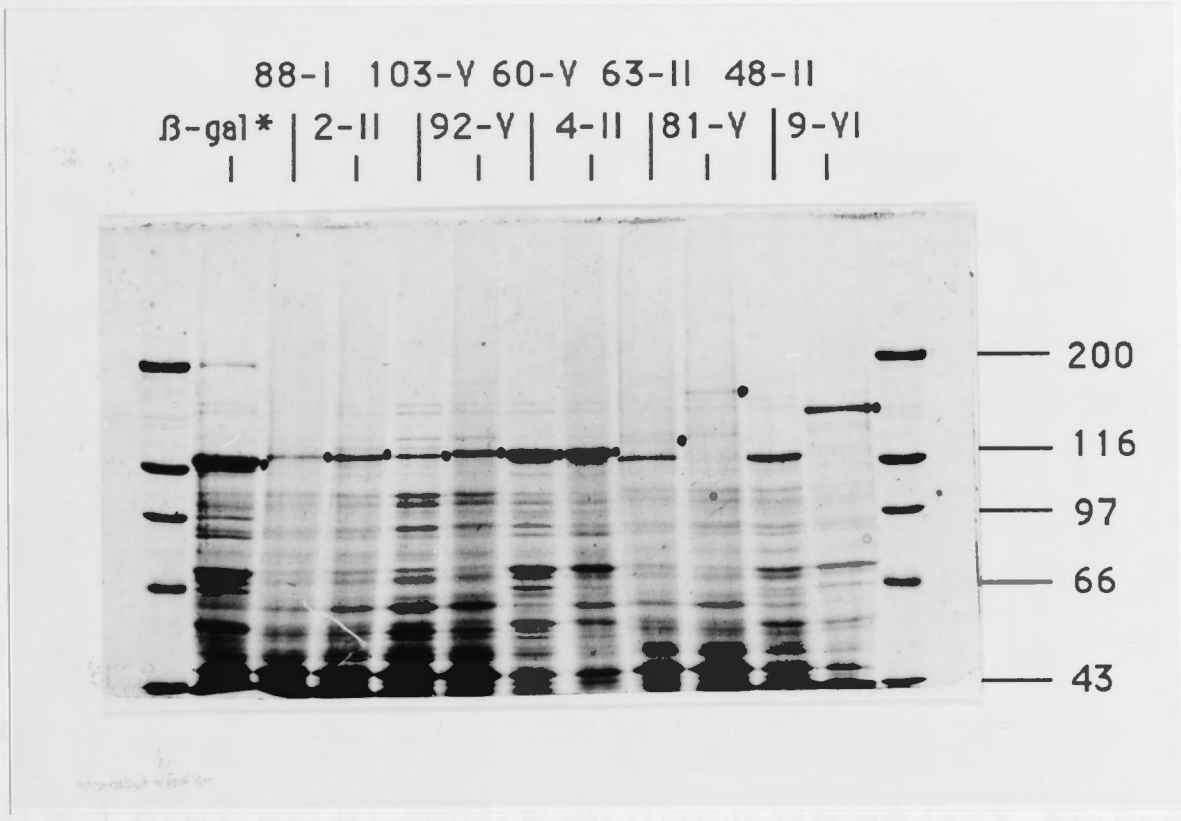


Figure 2.6 SDS-PAGE profiles of lysates of immunoreactive colonies. Lysates (1 ml) of clones 88-I, 2-II, 103-V, 60-V, 92-V, 4-II, 63-II, 9-VI, 81-V and 48-II were prepared from induced cultures (20 ml) by SDS lysis and sonication. Samples (1 µl) were incubated with ß-ME (5%; v/v) in SDS-PAGE sample buffer (15 µl final volume) at 90° for 2 min. The reduced samples were electrophoresed on SDS-7.5% PA gels at 200 volts for 45 min. The gels were then soaked in Coomassie Blue staining solution for 1 hour, destained and photographed. Size markers (SM; ≈0.5 µg each) were myosin (200K), ß-galactosidase (116K), rabbit muscle phosphorylase b (97K), bovine serum albumin (66K) and hen egg white ovalbumin (43K). The positions of ß-gal* and fusion proteins are indicated by dots next to the protein bands. The ß-gal* band observed in clone 63-II was due to contaminating pEX plasmids.

normally undergo proteolytic cleavage in MVE-infected cells at the N-termini of NS1 and NS2A. However the observed mol. wt. of the 81-V fusion protein corresponded to the full length E-NS1-NS2A, suggesting that cleavage had not occurred. This is not surprising as signalase in the lumen of the RER is responsible for cleavage at the N-terminus of NS1 (see Chapter 1).

The fusion protein from clone 63-II, which contained three Sau3A fragments, was predicted to comprise 110 amino acids including part of NS2A. The observed size of 134K was in agreement with this prediction (Fig. 2.5).

Fusion proteins from clones 88-I, 2-II, 103-V, 60-V, 92-V, 11-VI and 4-II were all approximately 125K. From the predicted amino acid sequences, two types of fusion protein were expected (Fig. 2.5). Their sizes were similar: 47 amino acids from NS2A for 88-I, 2-II and 103-V and 45 amino acids from NS2A for 60-V, 92-V, 11-VI and 4-II. The immunoreactivity of clones 60-V, 92-V, 11-VI and 4-II suggested that an NS2A fragment was expressed despite the out-of-frame insertion. It is possible that the NS2A fusion protein was expressed by ribosome slippage at the junction between lacZ* coding region and the NS2A gene fragment . This phenomenon has been reported by Stanley (1983) using pEX-like vectors; some clones expressed two types of fusion protein resulting from translation of inserts in different reading frames. For clones 60-V, 92-V and 11-VI, the two types of fusion protein from translation in different reading frames would be indistinguishable by size.

In summary, there was a good correspondence between observed and predicted mol. wts. of the fusion proteins (see Table 2.1). Extensive degradation of the fusion proteins had apparently not occurred.

2.3.7 Summary

A Sau3A-partial digest of a cDNA clone of the 5' terminal half of the MVE genome was cloned into pEX plasmids to generate an MVE expression library. Eleven

recombinant plasmids which expressed fusion proteins of ß-gal* and MVE proteins were identified by screening with anti-MVE HIAF. These contained inserts ranging from ≈100 bp to 2.8 kb. Sequence analysis of these plasmids identified the inserts, from which it was deduced that fusion proteins containing MVE protein fragments prM-E, E, E-NS1-NS2A and NS2A were expressed. Three plasmids out of eleven expressed portions of the E gene while eight expressed an NS2A sequence. For all of these plasmids except one (p48-II, which contained the largest insert), fusion proteins were expressed in yields of 5-40 mg per litre of induced culture and appeared to be free from proteolytic degradation.

2.4 DISCUSSION

We have investigated the possibility of expressing MVE proteins in *E.coli* with the aim of obtaining a collection of MVE peptides which react with anti-MVE HIAF for antigenic analysis and for studies of functionally important regions. By incorporating Sau3A-digested MVE cDNA fragments coding for prM, E, NS1, NS2A, NS2B and NS3 in pEX vectors, it was hoped that recombinant pEX plasmids which expressed MVE protein fragments, particularly of the E protein, would be generated.

The construction of an MVE expression library using the pEX vectors

Colonies producing fusion proteins were identified by immunoscreening with anti-MVE HIAF. The screening procedure was reliable and identified positive clones with ease even when the frequency of immunoreactive colonies was low. The identity of fusion proteins was deduced by DNA sequence determination and from the mol. wts. of fusion proteins. The eleven positive clones characterized in this study contained fragments of the prM, E, NS1 and NS2A genes. Clones 9-VI, 81-V and 48-II expressed parts of E. Colonies expressing NS2B and NS3 epitopes were not isolated. Whether or not this was due to the cloning or to the screening reagent (HIAF), is not known.

It is notable that nine out of the eleven clones analysed expressed the NS2A peptide Asp 7-Asp 53, either alone or in conjunction with other peptides. Fragments derived from cleavage at certain Sau3A sites (MVE nucleotides 2255, 3547, 3685 and pMT nucleotides 1490 and 1529; Fig. 2.4) occurred more than once in recombinant plasmids. Thus preferential cleavage of p2/1/22 at some SauA sites occurred and was responsible for the occurrence of the NS2A gene fragment in most of the immunoreactive colonies. The existence of such preferred cleavage sites imposes a restriction on the 'partial digest' approach to obtaining a random set of overlapping protein fragments expressed from the pEX plasmids. This limitation is likely to apply no matter what restriction enzyme is used to generate the partial digest. Certainly in our hands we have not obtained a series of E protein fragments for systematic epitope mapping. In Chapter 3, we adopt a directed approach to expressing the unrepresented parts of the E gene in the pEX vector, and use constructs generated in this manner to define functional determinants in E.

Yields and stability of MVE fusion proteins

Degradation of fusion proteins was not extensive as judged by a comparison of predicted and actual sizes of proteins in SDS-PAGE profiles. However degradation by end-trimming could not be ruled out as we could not detect small differences in mol. wts. or small degradation products (less than 40K). Stanley (1983) and Stanley and Luzio (1984) also reported that the insoluble ß-gal* fusion proteins from expression of recombinant pEX constructs were relatively stable.

A range of yields was observed. Generally, larger MVE fragments were expressed poorly. The fusion protein in 81-V comprising E-NS1-NS2A (476 amino acids) was poorly expressed. For 48-II, an even larger fusion protein comprising prM-E (573 amino acids) was not detectable by staining, and the ß-gal* protein was observed instead. As the colony was immunoreactive, the MVE fragment was expressed. Two explanations may account for this. The intact fusion protein may have been expressed in

trace quantities or been degraded into small fragments, some of which reacted with anti-MVE HIAF.

Certain 'foreign' sequences in viral proteins affect the yield of fusion proteins or the extent of degradation. It has been suggested that hydrophobic sequences in foreign proteins are toxic to bacteria, affecting the yield and stability of recombinant proteins (Yelverton *et al.*, 1983). This was observed in the expression of JE proteins as ß-galactosidase fusion proteins in *E.coli* : fusion proteins with the hydrophobic C-terminal domains of prM and E were not expressed in abundance (Mason *et al.*, 1987b). Although the two large, poorly expressed proteins from MVE clones 81-V and 48-II contained the hydrophobic C-termini of E and prM respectively, either the presence of hydrophobic sequences or the size of the protein could have accounted for the poor yield. The NS2A peptide expressed in several clones contained C-terminal hydrophobic sequences (see below); however the effect on yield was probably not significant as some of these clones expressed fusion proteins in abundance.

Reactivity of anti-MVE HIAF with fusion proteins

The differences in immunoreactivity between clones may result from the following factors. First, clones producing moderate to large amounts of fusion protein always showed strong immunoreactivity and thus quantity of protein may be a factor. The second factor is size: larger MVE peptides are likely, in a general sense, to contain more epitopes which react with anti-MVE HIAF than smaller peptides. This could explain the strong immunoreactivity of clone 81-V which expressed trace amounts of the second largest fusion protein. Similarly, clone 48-II did not express detectable amount of fusion protein but was still reactive with anti-MVE HIAF. The third factor is the inherent antigenicity and conformation of MVE components of the fusion proteins which could also affect immunoreactivity. It is likely that certain parts of MVE proteins are immunodominant and would react strongly with anti-MVE mouse HIAF. Although no information is available for MVE proteins, DEN-2 NS1 protein, expressed as

ß-galactosidase fusion proteins has been studied with polyclonal rabbit and mouse antisera. The N-and C-termini react strongly with mouse and rabbit sera respectively (Putnak *et al.*, 1988). The conformation of epitopes in MVE proteins prM, E and NS1 will also be influenced by formation of disulfide bonds, folding of discontinuous epitopes and glycosylation. These factors are important for the reactivity of some epitopes in TBE E (Guirakhoo *et al.*, 1989; Mandl *et al.*, 1989b). As the MVE fusion proteins are not Asn-glycosylated and only contain fragments of the MVE proteins, some epitopes may not be generated. Protein fragments of JE, DEN-1 and DEN-2 which are expressed in *E.coli* as fusion proteins react with some, but not all anti-E and anti-NS1 mAbs tested (Mason *et al.*, 1987b; Zuegel *et al.*, 1987; Putnak *et al.*, 1988) indicating that some viral epitopes are not present in the fusion proteins. Fusion proteins generated in our study were a non-random set, and did not allow analysis of immunodominant regions or of the conformation of MVE epitopes.

Application of fusion proteins to studies of MVE proteins

In addition to their value in mapping epitopes on E and NS1, fusion proteins have been used in the generation of specific antisera. Such antisera have been used in the isolation of YF proteins by immunoprecipitation and in the identification of NS2A, NS2B and NS4B (Chambers *et al.*, 1989). Specific antisera have also been employed in studies of the pathway of processing of YF polyproteins (Chambers *et al.*, 1990b). Such antisera may also be used to localize viral proteins during infection.

Some of the MVE fusion proteins generated in this study may be useful in the generation of antisera. The fusion protein carrying the NS2A peptide Asp 7-Asp 53 was most abundantly obtained in this study and may be useful in generating specific antisera for studying the expression and roles of the MVE NS2A and of a putative NS1-NS2A protein. NS1-NS2A in JE-infected cells was identified using a bacterial ß-galactosidase fusion protein containing an NS2A peptide (Mason *et al.*, 1987b). As there is identity between MVE and JE in the hydrophilic region between NS2A Asp 7

and Arg 31 (McAda *et al.*, 1987; Dalgarno *et al.*, 1986), and strong homology with WN, KUN in the same region (Castle *et al.*, 1986; Coia *et al.*, 1988), antisera generated against the MVE fusion protein may cross-react and allow comparative studies. However, this application of fusion proteins in studying protein processing was not pursued further in this thesis.

We have obtained fusion proteins from clones 9-VI, 81-V and 48-II which contain parts of E and could be considered for epitope mapping and for studies of functions of E such as interaction with cell receptors and fusion with membranes. However clone 81-V only expressed the C-terminus of E and 48-II appeared to be unstable. Clone 9-VI, which expressed fusion protein E146-429, is employed in epitope mapping studies in the following chapter.

CHAPTER 3

EPITOPE MAPPING ON MVE FUSION PROTEINS BY DELETION ANALYSIS AND IMMUNIZATION STUDIES IN MICE

3.1 INTRODUCTION

In this chapter we explore the possibility of mapping the epitopes of five neutralizing mAbs on MVE E protein fragments expressed in *E.coli*. To do this, we use deletion mapping, a method which has been valuable for a number of virus types (*Picornaviruses*, *Herpesviruses*, *Coronaviruses*; see Lenstra *et al.*, 1990) including JE and DEN-1 (Mason *et al.*, 1989; Zuegel *et al.*, 1987). In Chapter 2 we have demonstrated the expression of the fusion protein E146-429 in an immunologically reactive form using the pEX vector system. For the studies described in this chapter, an essential further step was to express the N-terminal portion of the E gene in the same system. Following this, the two construct could be subjected to deletion analysis to localize neutralization epitopes using mAbs.

Five neutralizing mAbs against MVE were available to us. Three of these mAbs, generated against MVE, had been characterized by Hawkes *et al.* (1988). The other two, which had in fact been generated against the closely related virus JE, were characterized by J. T. Roehrig (personal communication). The properties of these mAbs including subclass, titres in plaque reduction neutralization tests (PRNT) and HI assays, and cross-reactivity with flaviviruses in ELISA have been described (see Table 3.1). Each mAb shows a set of different activities and cross-reactivity patterns from the above tests. Analysis by competitive binding assays (CBAs) indicated that of the five epitopes defined by these mAbs, E-1c, E-1d, E-5b and E-8 are arranged in a continuum of four overlapping sites while E-7 appears to be spatially separate (J. T. Roehrig, personal communication). With one exception, there were no data available on the location, within the E protein sequence, of the epitopes defined by these mAbs. The exception was the description by S. H. Hartley and R. C. Weir (personal communication) that Ala 126 in E was part of the E-1c epitope. In this and the following chapter, studies which map the epitopes defined by these mAbs are reported.

TABLE 3.1

Physical and biological properties of neutralizing mAbs against MVE[a]

Epitope	mAb	Subclass	Cross-reactivity in ELISA[b]	Biological activity[c] HI titre[d]	N titre[e]	Protection[f]
E-1c	4B6C-2	IgG2a	MVE	1024	5120	MVE
E-1d	4B5A-2	IgG2a	MVE	<10	320	none
E-5b	4B3B-6	IgG2b	MVE, JE	<10	5120	MVE
E-7	6A4D-1	IgG1	MVE, JE, WN	<10	2048	MVE, JE
E-8	6B4A-10	IgG1	MVE, JE SLE, WN	<10	2048	MVE, JE, SLE

[a] Information on physical and biological properties of anti-E-1c, E-1d and E-5b mAbs are from Hawkes *et al.* (1988). Physical and biological properties of anti-E7 and E-8 mAbs were provided by Dr J. T. Roehrig (personal communication).

[b] Cross-reactivity was determined by antigen capture ELISA (Hawkes *et al.*, 1988). A large battery of antigens including those from MVE, JE, WN, SLE, Alfuy (ALF), YF and DEN-1 were tested; those showing positive reactivity are listed.

[c] For biological assays antibodies were purified from ascitic fluids by ammonium sulphate precipitation and Protein A-Sepharose column chromatography and standardized to 100 µg/ml (Hawkes *et al.*, 1988).

[d] Determined against MVE.

[e] Neutralization (N) titres of mAbs are reported as reciprocals of 70% end-points in plaque reduction neutralization tests using 50-100 PFU per test.

[f] Protection of 21-day old Swiss outbred mice by the mAbs was assessed by injecting a range of doses of antibodies (0.01 µg-100 µg) into the tail vein of animals 24 hours before ip challenge with 500 ipLD50 of virus. Anti-E-1c was the most protective antibody tested, requiring 0.1 µg to protect 50% of MVE-challenged mice. Other mAbs which protected mice were anti-E-5b, E-7 and E-8 (requiring 5-100 µg mAbs to protect >10% of challenged mice). Viruses which could be protected against in the assays are listed (J. T. Roehrig, personal communication).

Another approach to defining functional domains in the E protein is to determine the capacity of various E fusion proteins to induce reactive antibodies in mice and to assay the properties of such antibodies. Accordingly, in this chapter we explore the capacity of fusion proteins containing large MVE E protein fragments to generate antisera in mice which react with MVE in ELISA and which neutralize virus infectivity.

3.2 MATERIALS AND METHODS

3.2.1 Plasmids and bacterial strains

See Materials and Methods, Chapter 2. M13 mp8 was used as RF DNA (New England Biolabs); sequence data are from the manufacturer's catalogue.

3.2.2 Cells and virus

African green monkey kidney (Vero) cells were grown at 37° in medium M199/lactalbumin hydrolysate (M199/LAH) supplemented with 10% bovine serum.

Working stocks of MVE-1-51 were prepared by passing MVE-infected suckling mouse brain homogenates once in Vero cells (MOI≈1) and harvesting culture supernatants. The titres on Vero cell monolayers were generally 10^6 to 10^7 PFU/ml. Purified MVE, provided by Dr R.C. Weir (Biochemistry Department, Faculty of Science, Australian National University), was prepared from infected SW13 cell supernatants by precipitation with polyethylene glycol and was banded in a glycerol-tartrate gradient (Lobigs *et al.*, 1986).

3.2.3 Subcloning MVE cDNA into the pEX plasmids

Procedures for extraction of plasmid DNA, RE and sequence analysis of plasmid DNA, transformation of *E.coli* and preparation of vector DNA for ligation were as described in Chapter 2.

To generate blunt-ends, DNA fragments (1 µg) were incubated (37°, 30 min) with T4 DNA polymerase (0.2 units; Boehringer) in the presence of dGTP, dATP, dTTP and dCTP (0.5 mM each), 30 mM Tris-HCl (pH 8), 60 mM potassium acetate, 10 mM

magnesium acetate, 0.5 mM dithiothreitol (DTT; Boehringer) and 0.1 mg/ml bovine serum albumin. The enzyme reaction was stopped by incubation at 70° for 10 min.

For ligation, vector DNA (≈0.1 µg), digested with restriction enzymes and blunt-ended, was mixed with MVE cDNA fragments in a molar ratio of 4:1 in a final volume of 10 µl containing 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 20 mM DTT, 1 mM ATP, 50 µg/ml bovine serum albumin and T4 DNA ligase (100 units; New England Biolabs). Incubation was at 16° for 16 hours.

M13 mp8 was used to 'adapt' the ends of a TaqI MVE cDNA fragment (see Results) for subcloning into the polylinker region of pEX. M13 mp8 plasmid (0.3 µg), which contains a unique AccI site in the polylinker was incubated with AccI (5 units) for 1 hour at 37° and 10 min at 70°. The digested M13 DNA (0.15 µg) and the TaqI restriction fragment (≈70 ng) were ligated using T4 DNA ligase (100 units) overnight at 16°. The ligation mixture was then incubated at 70° for 10 min, BamHI and PstI (5 units each) were added and incubation continued at 37° for 1 hour. The TaqI fragment with BamHI and PstI cohesive ends was isolated on 1% low melting temperature (LMT) agarose (Bio-Rad).

Restriction fragments were stained with ethidium bromide (0.5 µg/ml). Gel slices containing DNA fragments were excised under UV illumination (wavelength = 380 nm) and melted in two volumes of 5 mM TE buffer containing 5 mM Tris-HCl (pH 8) and 0.5 mM EDTA at 65° for 5 min. The gel mixture was extracted with phenol (x2), phenol/chloroform and chloroform (Maniatis *et al.*, 1982). DNA was precipitated with sodium acetate (0.3 M, final concentration, pH 5.2) and ethanol (2 volumes) and dissolved in 5 mM TE buffer at pH 7.4.

3.2.4 Anti-MVE HIAF and anti-E mAbs

Polyclonal antibody against MVE was anti-MVE-1-51 HIAF (Chapter 2). Five mAbs against MVE were provided by Dr J.T. Roehrig (Division of Vector-Borne Viral

Diseases, Fort Collins, Colorado, USA). Table 3.1 lists their physical and biological characteristics. Three features of these mAbs are of interest to our work. First, all of the mAbs neutralize MVE infectivity. Second, the mAbs differ in ELISA cross-reactivity with other flaviviruses; mAbs to E-1c and E-1d are specific for MVE while mAbs to E-5b, E-7 and E-8 cross-react with other members of the JE complex. Third, mAb to E-1c is the only one showing both neutralization and HI activities; it is also the most effective in protecting mice from ip challenge with MVE. The ELISA titres were determined for anti-E-1c ($1x10^6$), anti-E-1d ($5x10^5$), anti-E-5b ($3x10^5$), anti-E-7 ($6x10^4$) and anti-E-8 ($5x10^5$).

3.2.5 Immunological screening and induction of fusion proteins

See Materials and Methods, Chapter 2.

3.2.6 SDS-PAGE and immunoblotting of fusion proteins

Fusion proteins were tested for reactivity with antibodies by either immunoblotting after SDS-PAGE or they were dot-blotted on nitrocellulose directly as lysates. To transfer proteins to nitrocellulose, SDS-PA gels containing separated proteins (Materials and Methods, Chapter 2) were soaked in blotting buffer (200 mM glycine, 10 mM Tris-HCl, pH 8.3, 20% methanol) for 15-30 min. Proteins were transferred electrophoretically to nitrocellulose (Towbin *et al.*, 1979) in blotting buffer at 4° for 1 hour. A Transblot apparatus (Bio-Rad) and a Bio-Rad Power Supply (Model 250/2.5) with settings at 0.15 Amp (65-75 volts) were used. For dot-blotting, bacterial lysates (1 µl) containing ≈1 µg fusion protein (in SDS-PAGE sample buffer without the bromophenol blue dye) were dotted on nitrocellulose. Membranes were rinsed with wash buffer (WB; 0.05% Tween-20 in PBS), soaked for 30 min in WB containing 5% non-fat milk powder, rinsed (x3) in WB, incubated with anti-MVE HIAF (1/800 dilution) for 1 hour or mAbs (1/400 dilution) for 16 hours, rinsed (x3) in WB and incubated for 1 hour with peroxidase coupled goat anti-(mouse IgG) (1/1500 dilution;

Bio-Rad). Detection was with 4-chloro-1-naphthol and hydrogen peroxide as described for immunoscreening (Chapter 2).

3.2.7 Purification of fusion proteins (M. J. Howell, personal communication)

E.coli cells containing pEX or recombinant plasmids were grown at 30° in LB medium containing ampicillin (100 µg/ml) and kanamycin (40 µg/ml) with constant agitation until stationary phase was reached. Cells were diluted in fresh medium to an O.D. of 0.05 at 630 nm. Incubation was continued at 30° for 1 hour and at 42° for 2 hours. Cells were collected by centrifugation at 8000 rpm (Sorvall SS-34 rotor) for 10 min, resuspended in 0.9% NaCl to 1/20th the original volume and lysed by three cycles of freezing in liquid nitrogen and thawing at 30°. Lysozyme was added to 125 µg/ml and the extract held on ice for 20 min followed by another cycle of freezing and thawing. The lysates were then sonicated for 30 seconds and centrifuged for 15 min in an Eppendorf centrifuge at 4°. The resulting pellets, which were enriched in fusion proteins, were resuspended in 8 M urea (Ultrapure; Sigma) in 10 mM Tris-HCl (pH 7.4). After clarification by centrifugation, fusion protein solutions were dialysed against PBS at 4° overnight and stored at -70°. Quantitation was by colorimetric analysis using the Bio-Rad protein assay kit. The protein standard used was bovine γ-gobulin (Bio-Rad).

3.2.8 Injection of mice with fusion proteins

Six to eight-week old outbred Swiss mice were injected in groups of five. Fusion proteins (10 µg each in 50 µl of PBS) were mixed with equal volumes of Freund's incomplete adjuvant (FIA; Difco), emulsified by passing though a syringe several times and injected ip. Booster injections were given four weeks and nine weeks after the initial injection. In some experiments, RIBI adjuvant containing monophosphoryl lipid A and trehalose dimycolate emulsion (RIBI Immunochem Research, Inc., Hamilton, Montana, USA) was used. A single dose for injection contained RIBI adjuvant (1 µg)

and fusion protein (10 μg) in 0.2 ml of PBS. A single booster infection was given five weeks after the initial injection. Animals were bled two weeks after the first injection and a week after boost. Serum samples were prepared by centrifugation (2 min) of coagulated blood in Eppendorf tubes and stored at -20°.

3.2.9 Partial purification of MVE for use as ELISA antigens

Virus from tissue culture supernatants was concentrated using polyethylene glycol (PEG 8000; Koch-Light Laboratories, Suffolk, England) for use as ELISA antigen (Cammack and Gould, 1986b). Monolayer cultures of Vero cells in 850 cm^2 roller bottles (Corning, New York, USA) were infected with MVE-1-51 (MOI≈10). At 24 hours p.i., M199/LAH medium was replaced with 50 ml of Eagle's minimal essential medium (EMEM) containing 2% foetal calf serum (FCS; Commonwealth Serum Laboratories, Melbourne, Australia), and incubation continued. At 48 hours p.i., culture supernatant was harvested and clarified by centrifugation for 10 min at 10,000 rpm (Sorvall GSA rotor). PEG (40% stock solution in NTE buffer: 0.12 M NaCl, 12 mM Tris-HCl, pH 8, 1 mM EDTA) was slowly added to a final concentration of 8% (w/v) with constant stirring at 4° for 2 hours. Virus was collected by centrifugation for 30 min at 10,000 rpm (Sorvall GSA rotor) and pellets drained and resuspended in 2 ml (per roller bottle of supernatant) sodium borate-saline (0.05 M borate, pH 9, 0.12 M NaCl). The concentrated virus solution was clarified by centrifugation and stored at -20°.

3.2.10 MVE plaque assays

Vero cell monolayers in 6-well plastic trays (TC grade, Linbro Scientific Inc., USA) were infected with virus (0.1 ml per well in duplicates) diluted in HBSS containing 20 mM HEPES (Sigma; pH 8). Adsorption was for 1 hour at 37° in an atmosphere of 5% CO_2 / 95% air with occasional shaking. Monolayers were overlaid with M199/LAH containing 1% agar (Difco), 0.02% (w/v) diethyl amino ethyl dextran (Sigma), 2% FCS and 4% bovine serum. Incubation was for 3 days at 37° as above;

plaques were counted after staining for 1 day with 0.015% (w/v) neutral red (Ajax) in 0.6% agar.

3.2.11 Enzyme-linked immunosorbent assay (ELISA)

Reactivity of immune sera with MVE or fusion proteins was examined by ELISA. Wells of microtitre plates (Titertek, Netherlands) or 96 well round bottom plates (Linbro) were coated for two hours at 37° with MVE (PEG-concentrated preparation) diluted to 10^6 PFU/ml in sodium borate-saline or with fusion proteins diluted to 10 µg/ml in sodium borate-saline. Plates were rinsed (x 3) with PBS containing 5% non-fat milk powder and incubated for 2 hours at 37° with sera diluted in PBS-5% milk powder. After rinsing (x 6) with PBS containing 0.05% Tween-20, bound antibodies were detected with goat anti-(mouse IgG) horseradish peroxidase diluted 1 in 600 in PBS-5% milk powder. Colour development was in citrate-phosphate buffer (61 mM citric acid, 77 mM Na_2HPO_4, pH4), 0.003% H_2O_2 and 1 mg/ml of 2,2'-Azinobis (3-ethylbenzthiazoline-sulfonic acid) (ABTS; Sigma). Plates were read at 410 nm using an automated plate reader (Dynatech). End-points were taken as the highest dilution which gave OD readings of >0.1. Controls were performed using sera collected from mice before injection.

3.2.12 Plaque reduction neutralization tests (PRNT)

Neutralization titres of anti-MVE mouse sera were determined by PRNT. Serial 2-fold dilutions of sera in 100 µl HBSS (pH 8) were incubated for 1 hour at 37° with 100-200 PFU of MVE-1-51 in 100 µl HBSS and the mixtures (200 µl) then assayed in duplicate by plaque formation. Control assays were performed using mouse sera collected prior to injection.

3.3 RESULTS

3.3.1 Subcloning and expression of the 5' half of the MVE E gene

Construction of recombinant plasmid

As described in the Introduction, our first aim was to subclone and express a section of the MVE E gene encoding the N-terminal portion of E. To do this the nucleotide sequence of E was scanned for suitable RE sites. It was noted that TaqI sites flanked a region encoding MVE peptide Asp 22-Val 272 (E_{22-272}; MVE peptides are denoted by numbers in subscript) which represents approximately the N-terminal half of E (251 amno acids; Fig. 3.1). The method used for cloning and expressing this region is described below (see also Fig. 3.2).

Plasmid p2/1/22 was digested with TaqI and the appropriate fragment (0.75 kb) isolated from LMT agarose and ligated into AccI-digested, dephosphorylated M13 mp8 (TaqI sites are compatible with the AccI site in M13 mp8). This was done in order to modify the 0.75 kb fragment for insertion into the pEX vector. The ligated products were digested with PstI and BamHI which cleave on either site of the AccI site and allowed insertion into PstI/BamHI-digested pEX vectors. The TaqI fragment with PstI and BamHI cohesive ends was isolated after electrophoresis on LMT agarose and ligated to BamH1- and Pst1-digested pEX vectors (Fig. 3.2). The ligation mixture was used to transform *E.coli* MC1061/pCI857. Ampicillin resistant colonies were screened with anti-MVE HIAF. pEX plasmid from one such clone was examined by digestion with BamHI and PstI and analysed by agarose gel electrophoresis. The size of the insert was ≈770 bp as expected (results not shown).

Size and yield of the MVE E fusion protein

The fusion protein expressed from the recombinant plasmid was examined by SDS-PAGE to determine its size and yield. The lysate was reduced with ß-ME, electrophoresed on an SDS-7.5% PA gel with size markers and stained with Coomassie

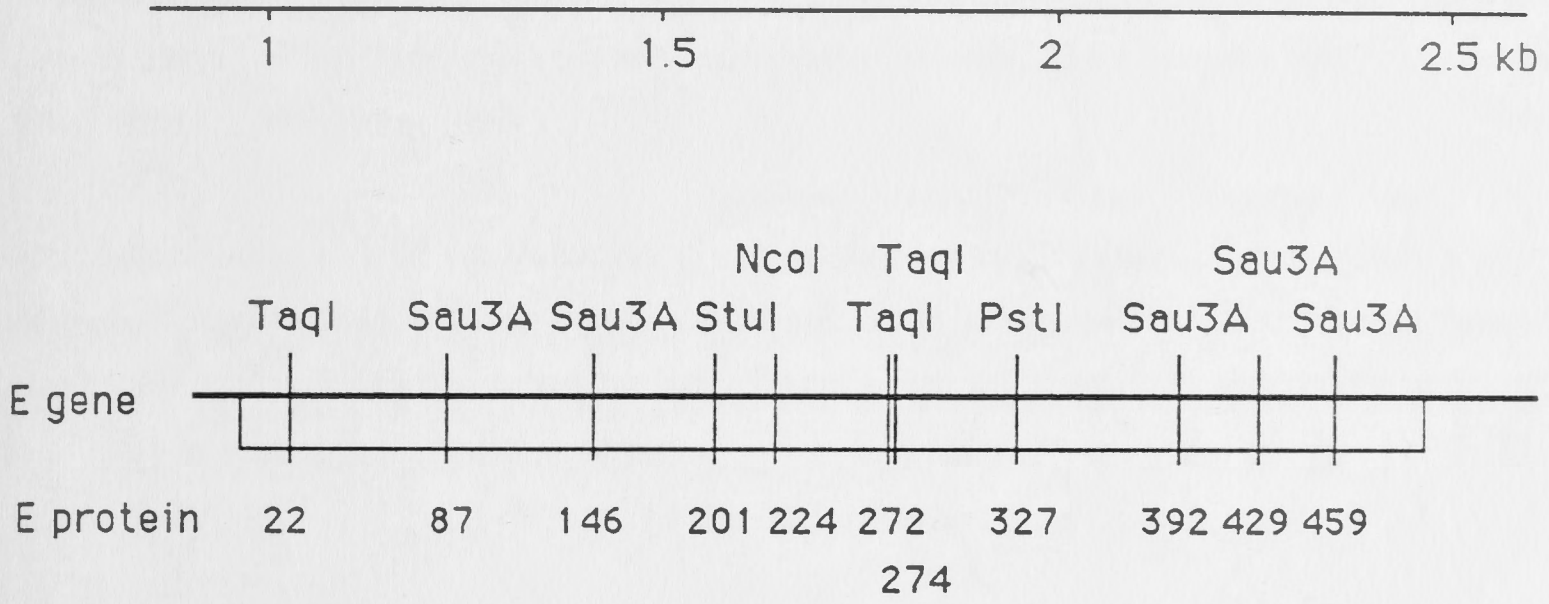
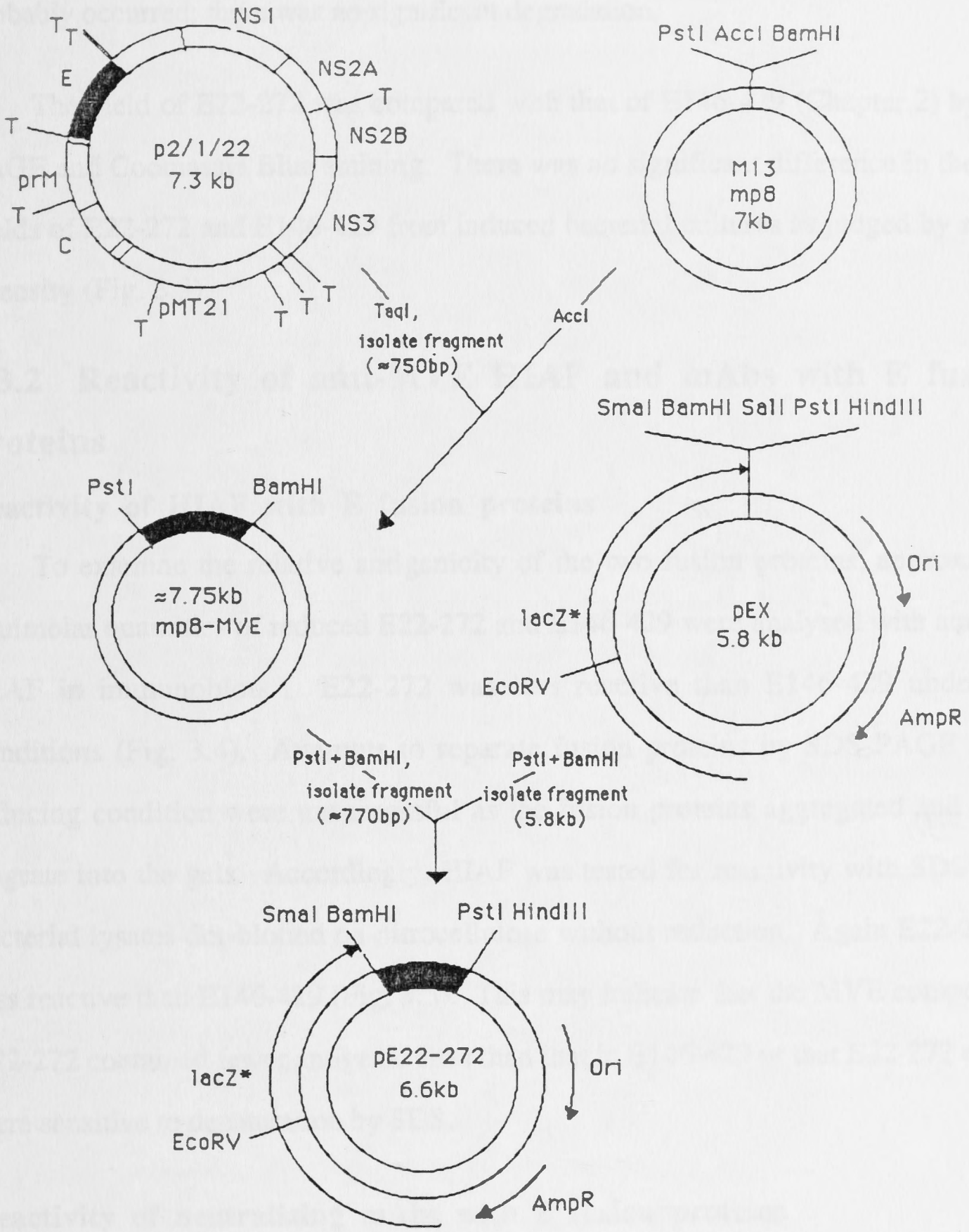


Figure 3.1 Restriction sites in the MVE E gene. Restriction sites which are relevant to the construction of recombinant plasmids are shown. There are three TaqI sites, five Sau3A sites and one each of NcoI, StuI and PstI sites in the coding region for E protein. Number below each site represent the corresponding amino acid positions in the MVE E protein (501 amino acids long). The scale indicates the location with respect to the 5' terminal nucleotide in the genome of MVE-1-51 (Dalgarno *et al.*, 1986).

Figure 3.2 Construction of plasmid pE22-272. p2/1/22 (1 μg; 7.3 kb) which contains nine TaqI sites was incubated with TaqI (5 units) at 60° for 1 hour. The digest was electrophoresed on 1% LMT agarose and a TaqI fragment (753 bp; 70 ng) from the E gene was identified, extracted and ligated to AccI-digested M13 mp8 followed by digestion with BamHI and PstI to create BamHI and PstI cohesive ends. pEX1, 2 and 3 plasmids (0.1 μg each) were incubated with PstI (5 units) and BamHI (5 units) in RE buffer (Materials and Methods, Chapter 2) containing 0.15 M NaCl at 37° for 1 hour. Calf intestinal alkaline phosphatase (1 unit) was added and incubation continued for 30 min. The mixture was electrophoresed on 1% LMT agarose to isolate the digested pEX vectors (5.8 kb). The pEX plasmids (0.2 μg) and TaqI fragment (15 ng), both possessing BamHI and PstI adhesive ends were incubated with T4 DNA ligase (100 units) at 16° overnight. The ligation mixture was used to transform competent MC1061/pCI857 cells. Screening was on ampicillin plates.


NS1
NS2A
NS2B
NS3
C
prM
E
T
p2/1/22
7.3 kb
pMT21
TaqI,
isolate fragment
(≈750bp)
PstI AccI BamHI
M13
mp8
7kb
AccI
SmaI BamHI SalI PstI HindIII
Ori
lacZ*
EcoRV
AmpR
pEX
5.8 kb
PstI
BamHI
≈7.75kb
mp8-MVE
PstI+BamHI,
isolate fragment
(≈770bp)
PstI+BamHI
isolate fragment
(5.8kb)
SmaI BamHI
PstI HindIII
pE22-272
6.6kb

Blue. The observed mol. wt. of the fusion protein (156K; Fig. 3.3) was close to the predicted mol. wt. of E22-272 (149K). Thus appropriate translation of the E peptide probably occurred; there was no significant degradation.

The yield of E22-272 was compared with that of E146-429 (Chapter 2) by SDS-PAGE and Coomassie Blue staining. There was no significant difference in the molar yields of E22-272 and E146-429 from induced bacterial cultures as judged by staining intensity (Fig. 3.3).

3.3.2 Reactivity of anti-MVE HIAF and mAbs with E fusion proteins

Reactivity of HIAF with E fusion proteins

To examine the relative antigenicity of the two fusion proteins, approximately equimolar quantities of reduced E22-272 and E146-429 were analysed with anti-MVE HIAF in immunoblots . E22-272 was less reactive than E146-429 under these conditions (Fig. 3.4). Attempts to separate fusion proteins by SDS-PAGE in non-reducing condition were unsuccessful as the fusion proteins aggregated and did not migrate into the gels. Accordingly, HIAF was tested for reactivity with SDS-treated bacterial lysates dot-blotted on nitrocellulose without reduction. Again E22-272 was less reactive than E146-429 (Fig. 3.5). This may indicate that the MVE component in E22-272 contained fewer antigenic sites than that in E146-429 or that E22-272 epitopes were sensitive to denaturation by SDS.

Reactivity of neutralizing mAbs with E fusion proteins

To examine the feasibility of using immunoblotting to assess the reactivity and specificity of the five mAbs, purified MVE was electrophoresed, with and without prior reduction with ß-ME, on SDS-20% PA gels, transferred to nitrocellulose and analysed with anti-E-1c, E-1d, E-5b, E-7 and E-8 mAbs. All five mAbs reacted with E in immunoblots of unreduced MVE (Fig. 3.6), indicating that the corresponding neutralization epitopes are located in E and remain reactive during the procedure used.

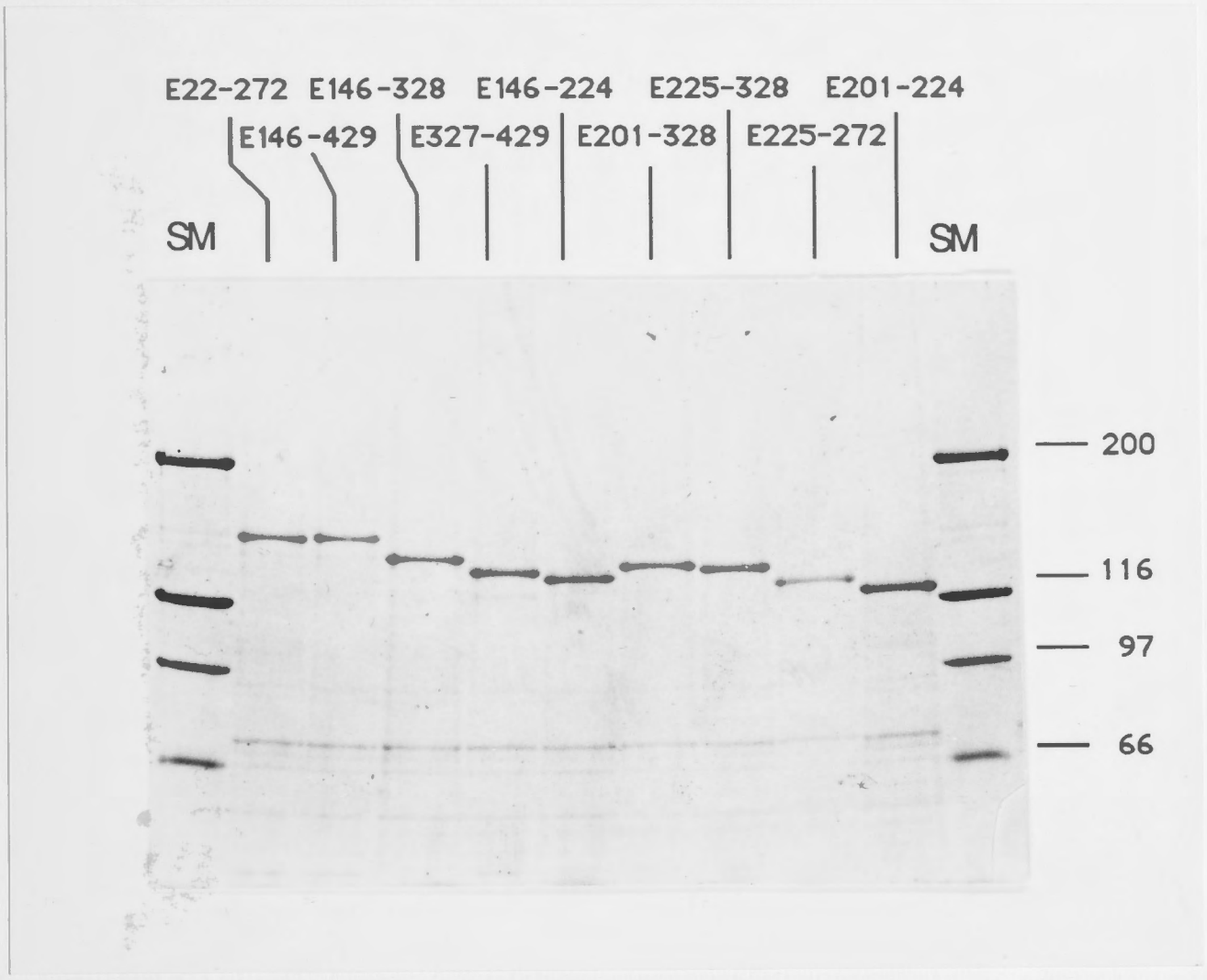


Figure 3.3 Coomassie blue stained SDS-PAGE profiles of colonies expressing MVE E fusion proteins. Immunoreactive colonies which were expected to express ß-gal* and the fusion proteins E22-272, E146-429, E146-328, E327-429, E201-328, E146-224, E225-328, E225-272 and E201-224 (see Table 3.2 for abbreviations) were induced for fusion protein synthesis in culture. Bacterial lysates (0.25 ml) were prepared from induced cultures (5 ml). Samples (1 µl) were incubated with ß-ME and SDS-PAGE sample buffer in 90° water bath for 2 min and electrophoresed on an SDS-7.5% PA gel. Proteins were detected by Coomassie Blue staining. The mol. wt. standards are myosin (200K), ß-galactosidase (116K), rabbit muscle phosphorylase b (97K), hen egg white ovalbumin (67K) and bovine serum albumin (43K).

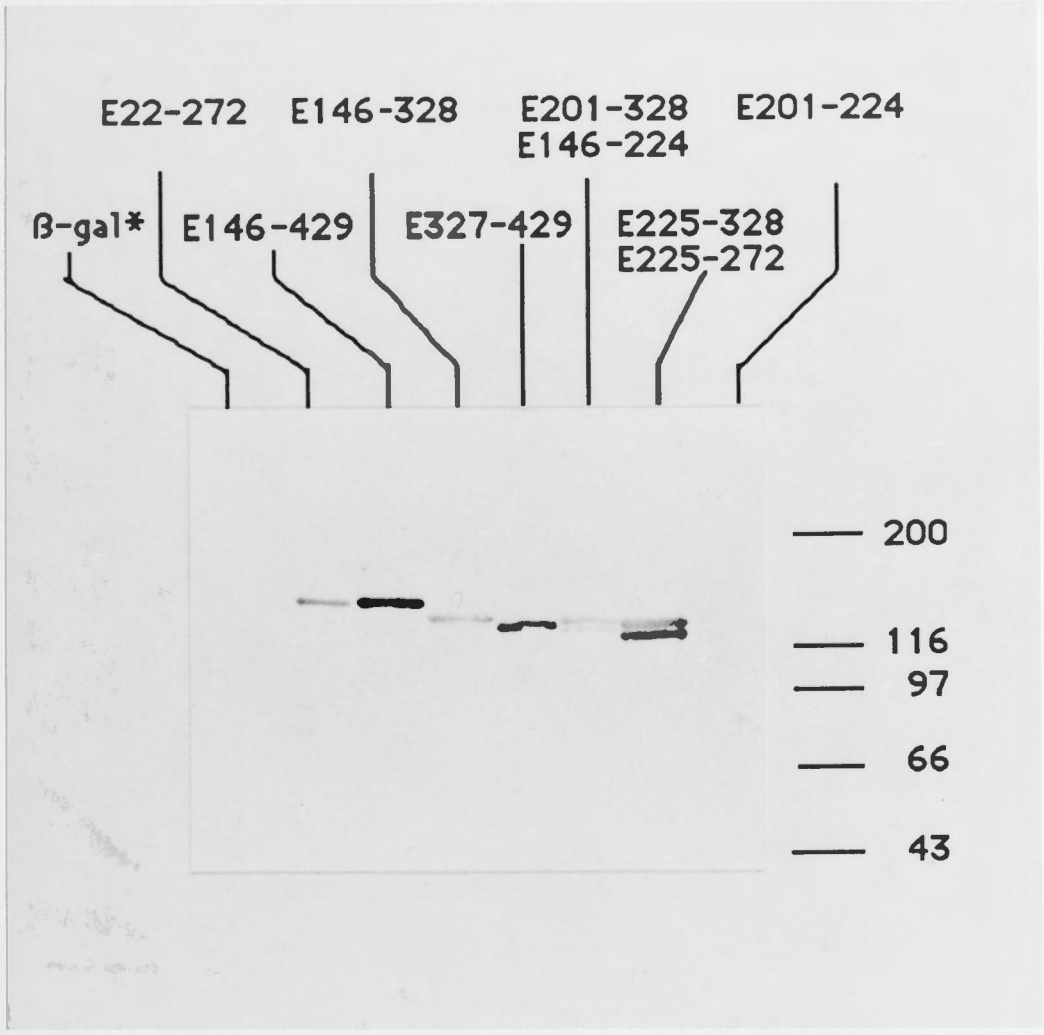


Figure 3.4 Immunoblot analysis of MVE E fusion proteins using anti-MVE HIAF. Bacterial lysates containing ß-gal* and fusion proteins E22-272, E146-429, E146-328, E327-429, E201-328, E146-224, E225-328, E225-272 and E201-224 (≈0.5 µg each) were electrophoresed on an SDS-7.5% PA gel after incubation with ß-ME for immunoblot analysis. Protein blots were detected with anti-MVE HIAF at dilution of 1 in 800 (1 hour incubation). Positions of size markers (in kilodaltons) are indicated.

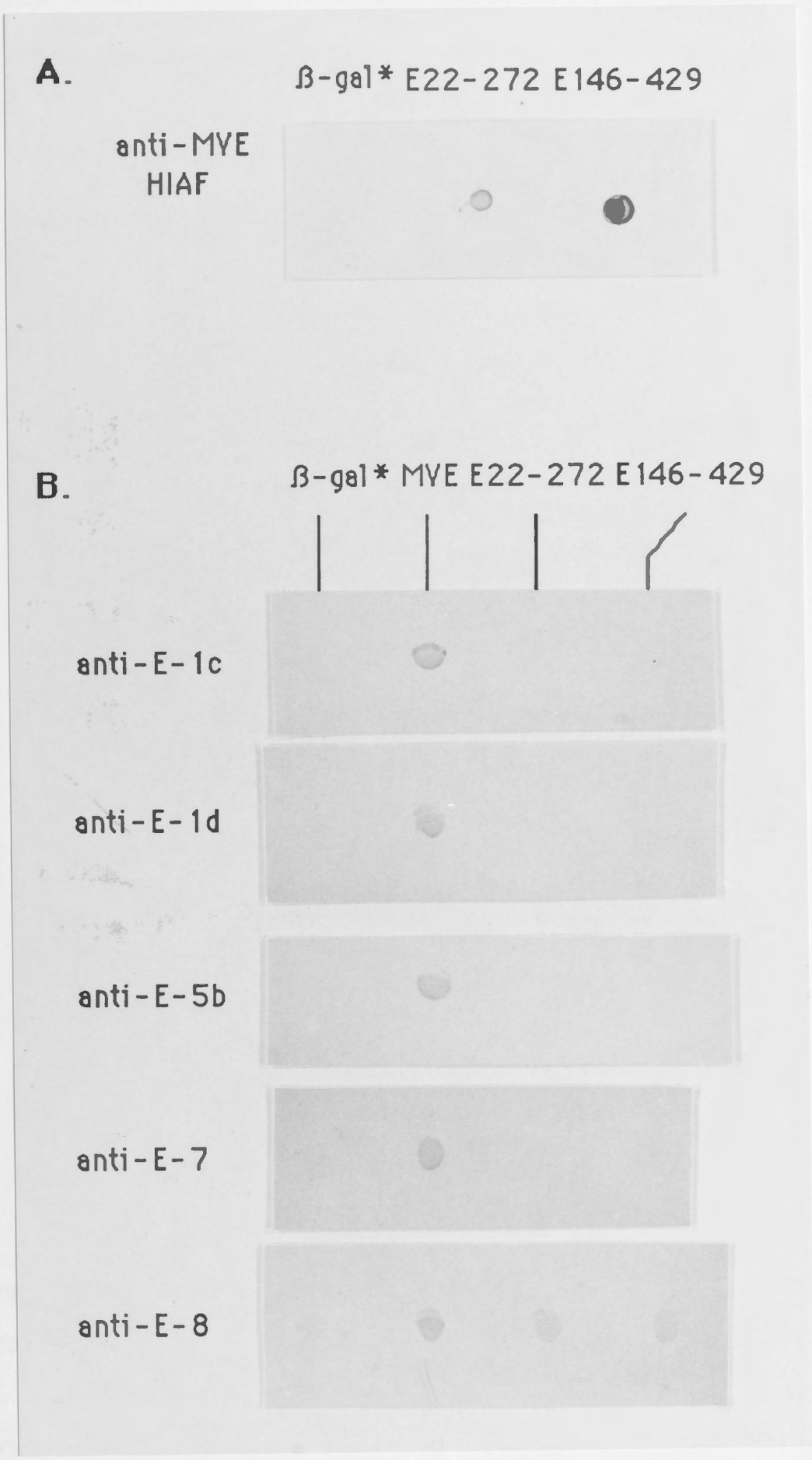


Figure 3.5 Reactivity of anti-MVE HIAF and anti-MVE mAbs with fusion proteins E22-272 and E146-429. Bacterial lysates containing ß-gal* , E22-272 and E146-429 (1 µg each) in PBS (final volume of 0.1 ml) were dot-blotted on nitrocellulose. The nitrocellulose membranes were soaked in 5% milk powder for 30 min and reacted with anti-MVE HIAF (diluted 1 in 800; 1 hour incubation) or anti-E-1c, E-1d, E-5b, E-7 and E-8 mAbs (diluted 1 in 500; overnight incubation). Controls were purified MVE containing ≈0.3 µg of E protein.

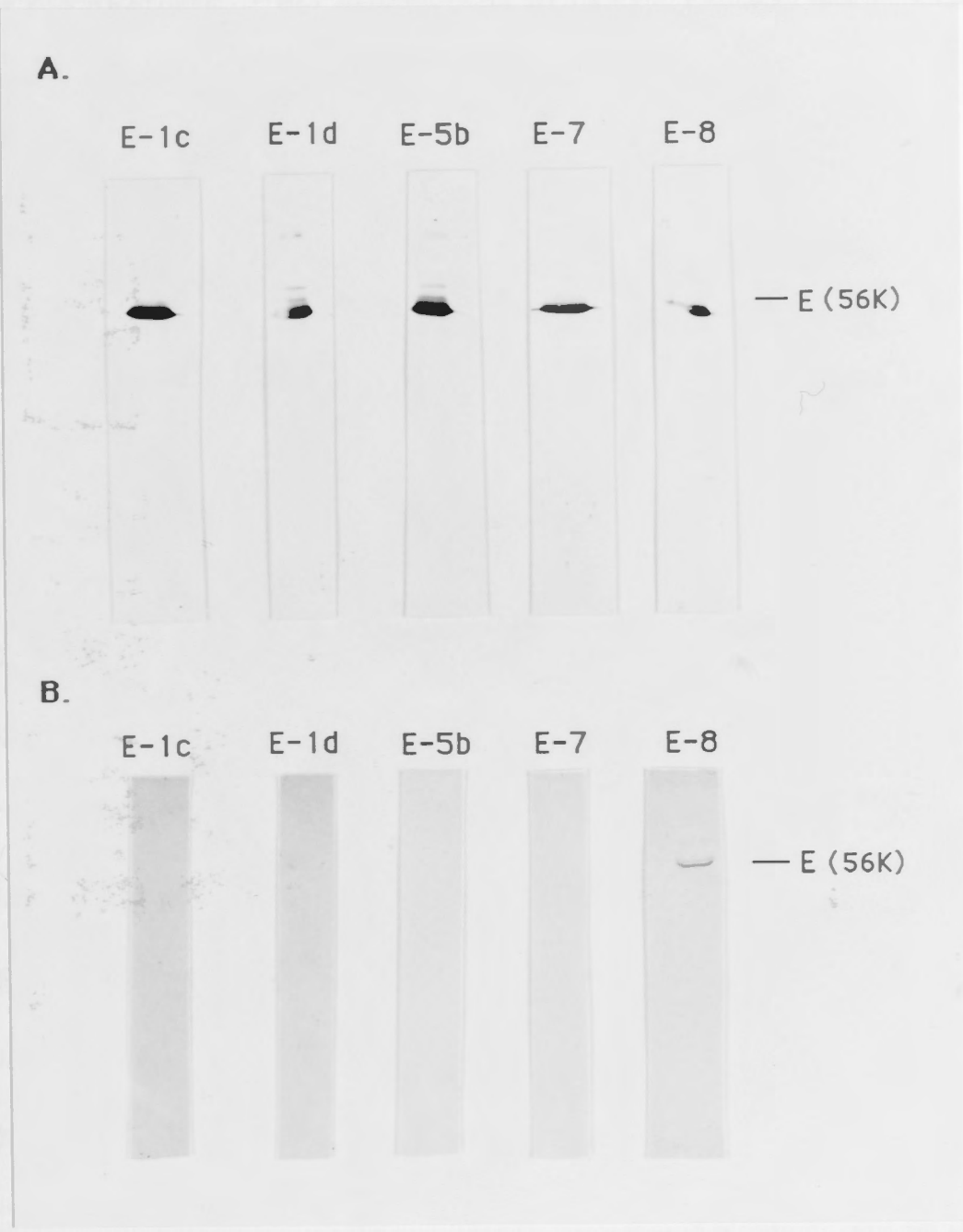


Figure 3.6 Immunoblot analysis of reduced and unreduced MVE proteins using anti-E mAbs. (A) Purified MVE containing ≈0.2 μg E was diluted in SDS-PAGE sample buffer at 4° and electrophoresed on an SDS-20% PA gel at 200 volts for 45 min. Proteins in the gel were transferred to nitrocellulose and detected with anti-E-1c, E-1d, E-5b, E-7 and E-8 mAbs at dilutions of 1 in 500 (overnight incubation at room temperature).

(B) Purified MVE containing ≈0.2 μg E was incubated with ß-ME at 90° for 2 min and electrophoresed on an SDS-20% PA gel as above. Proteins were transferred to nitrocellulose and detected by mAbs as above. The MVE E protein (56K) is indicated by arrow.

However, only anti-E-8 showed reactivity with reduced MVE E protein (Fig. 3.6), indicating that epitopes E-1c, E-1d, E-5b and E-7 are lost on reduction. These results indicated that the analysis of the reactivities of mAbs against the E-1c, E-1d, E-5b and E-7 epitopes would require unreduced fusion proteins while anti-E-8 mAb would react with both unreduced and reduced fusion proteins.

The reactivity of the two ß-gal*-E fusion proteins with mAbs was analysed by dot-blotting bacterial lysates on nitrocellulose without reduction. Anti-E-8 reacted with E22-272 and E146-429 but anti-E-1c, E-1d, E-5b and E-7 mAbs showed no reactivity with either of the unreduced fusion proteins (Fig. 3.5).

Reduced E22-272 and E146-429 were electrophoresed on SDS-PA gels for immunoblotting using anti-E-8 mAb. Both fusion proteins were reactive to similar extents after reduction (Fig. 3.7). When equimolar amounts of MVE E protein and fusion proteins (E22-272, E146-429) were compared, the reactivity of fusion proteins was only slightly less than E (Fig. 3.5). For this experiment, 1 µg of MVE E protein (56K) was taken as equivalent to approximately 3 µg of E22-272 or E146-429. Hence both fusion proteins appeared to carry the E-8 epitope.

3.3.3 Mapping epitope E-8 on the MVE fusion proteins

Construction of plasmids for deletion mapping

The above results indicated that fusion proteins E22-272 and E146-429 could be used to map the E-8 epitope. Since they both showed reactivity, the simplest explanation was that the binding site was located in the overlapping region between Ser 146 and Val 272, i.e., in a stretch of 127 amino acids encoded in ≈380 bp. To more closely define this region, recombinant pEX plasmids containing various segments of the MVE E gene were derived by subcloning from pE22-272 or pE146-429, and used to express fusion proteins for analysis of reactivity with anti-E-8 mAb. In total, seven different recombinant plasmids were constructed. Fig. 3.8 shows the inserts and corresponding MVE E peptides encoded by these plasmids.

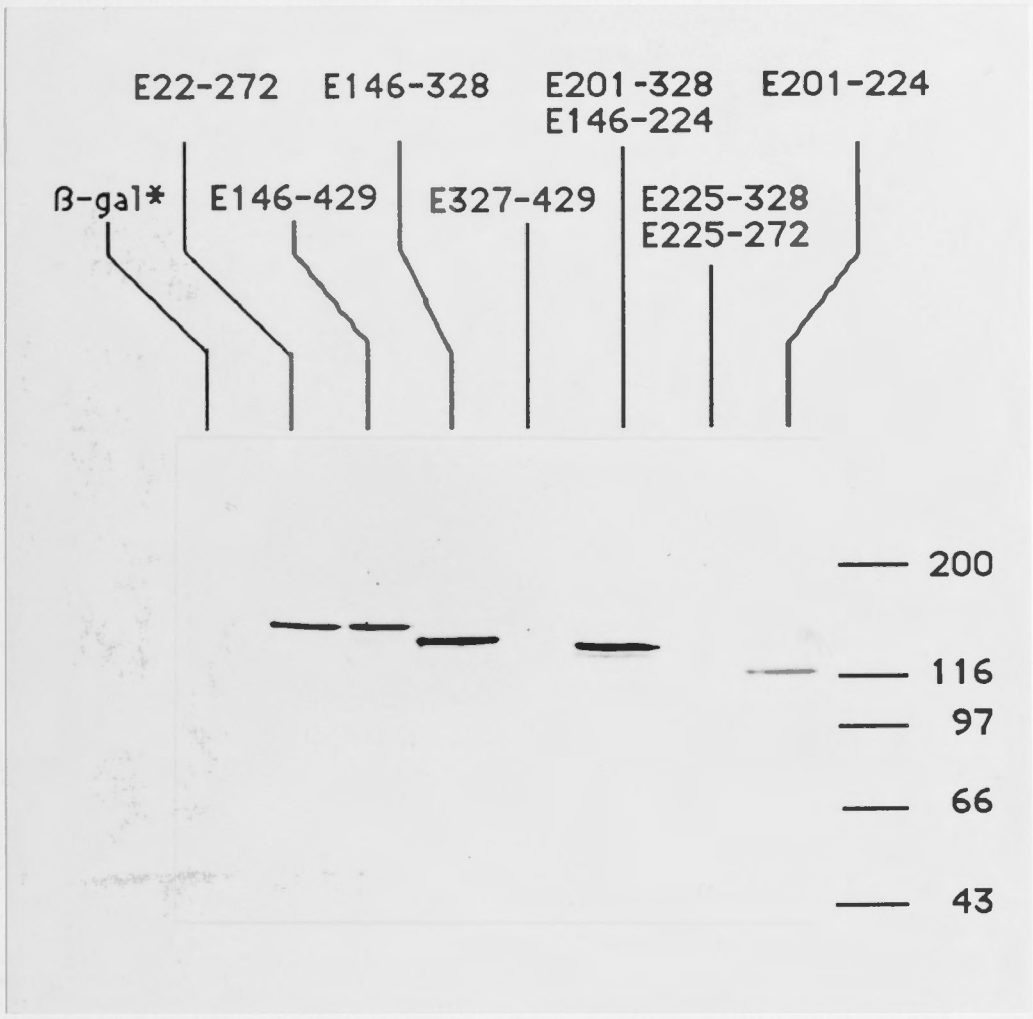


Figure 3.7 Immunoblot analysis of reduced fusion proteins (see Table 3.2) using anti-E-8 mAb. Bacterial lysates containing ß-gal* and fusion proteins (≈0.5 µg each) were electrophoresed on an SDS-7.5% PA gel after incubation with ß-ME for immunoblot analysis. Protein blots were detected with anti-E-8 mAb at dilution of 1 in 500 (overnight incubation). Positions of size markers (in kilodaltons) are indicated.

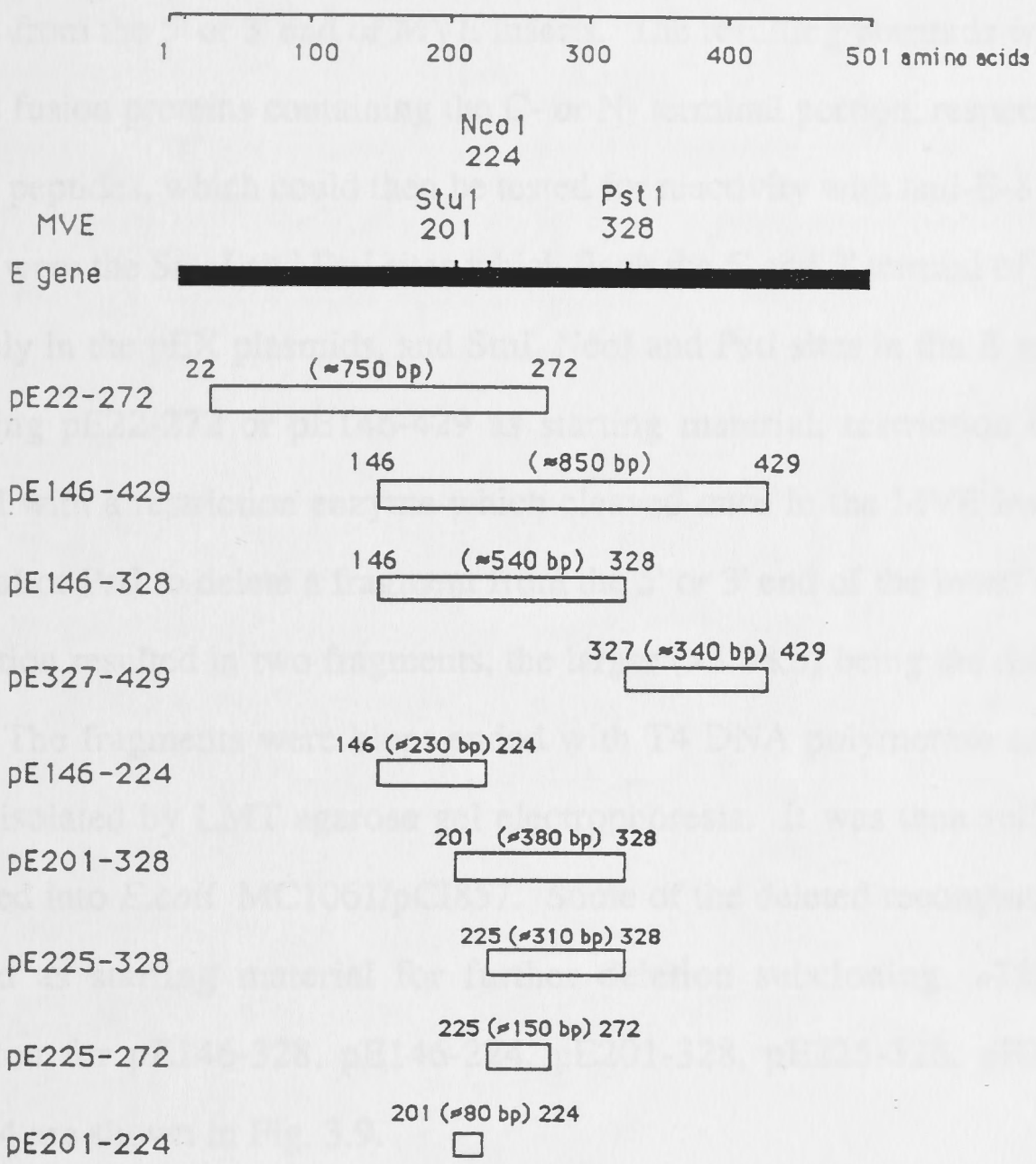


Figure 3.8 Identity of E gene inserts in recombinant plasmids. The solid bar represents the MVE E gene. The scale indicates the distance (in amino acids) from the N-terminal amino acid of the MVE E protein (501 amino acids). The three unique restriction sites used in construction of recombinant plasmids and the corresponding amino acid positions in the E protein are shown. The open bars represent the E gene inserts in recombinant plasmids. The sizes of these inserts are shown in brackets. The amino acid positions in E corresponding to the ends of these inserts are shown on either side.

The strategy for construction of these plasmids, except for pE327-429, relied on the presence of unique restriction sites in recombinant plasmids for deleting restriction fragments from the 5' or 3' end of MVE inserts. The resulting plasmids were expected to express fusion proteins containing the C- or N- terminal portion, respectively, of the original E peptides, which could then be tested for reactivity with anti-E-8. Restriction sites used were the SmaI and PstI sites which flank the 5' and 3' termini of MVE inserts respectively in the pEX plasmids, and StuI, NcoI and PstI sites in the E gene (see Fig. 3.9). Using pE22-272 or pE146-429 as starting material, restriction digests were performed with a restriction enzyme which cleaved once in the MVE insert and with either SmaI or PstI to delete a fragment from the 5' or 3' end of the insert respectively. The digestion resulted in two fragments, the larger (>5.8 kb) being the desired deleted plasmid. The fragments were blunt-ended with T4 DNA polymerase and the larger fragment isolated by LMT agarose gel electrophoresis. It was then self-ligated and transformed into *E.coli* MC1061/pCI857. Some of the deleted recombinant plasmids were used as starting material for further deletion subcloning. The details of construction for pE146-328, pE146-224, pE201-328, pE225-328, pE225-272 and pE201-224 are shown in Fig. 3.9.

Plasmid pE327-429 was constructed differently. A fragment coding for peptide $E_{327\text{-}429}$ (≈350 bp; Fig. 3.1) was isolated from pE146-429 after PstI digestion and LMT agarose gel electrophoresis (Fig. 3.10), and ligated to PstI-digested, dephosphorylated pEX 1, 2 and 3 plasmids. The ligation mixture was used to transform MC1061/pCI857 cells. Ampicillin resistant colonies were screened using anti-MVE HIAF. Plasmids were extracted from immunoreactive colonies and examined by RE analysis to estimate size of inserts. A unique EcoRV site located 1.9 kb upstream from the 3' terminus of lacZ* (Fig. 2.1) and the PstI or NcoI sites which flank inserts at the 3' end (Fig. 3.9) were used to cut out the inserts in full. Fragment sizes were determined by agarose gel electrophoresis. The recombinant plasmids each contained an insert from the E gene of the expected size (results not shown).

Figure 3.9 Construction of recombinant plasmids for deletion mapping.

pE146-328: pE146-429 (1 μg) was incubated with PstI (5 units) at 37° for 1 hour. The digest was electrophoresed on 1% LMT agarose, two fragments were observed (6.3 kb and ≈300 bp). The larger fragment (≈0.5 μg) was extracted and self-ligated by T4 DNA ligase (100 units) at 16° overnight. The ligation mixture was then used to transform MC1061/pCI857. Colonies carrying the recombinant plasmid were selected by immunoscreening using anti-MVE HIAF.

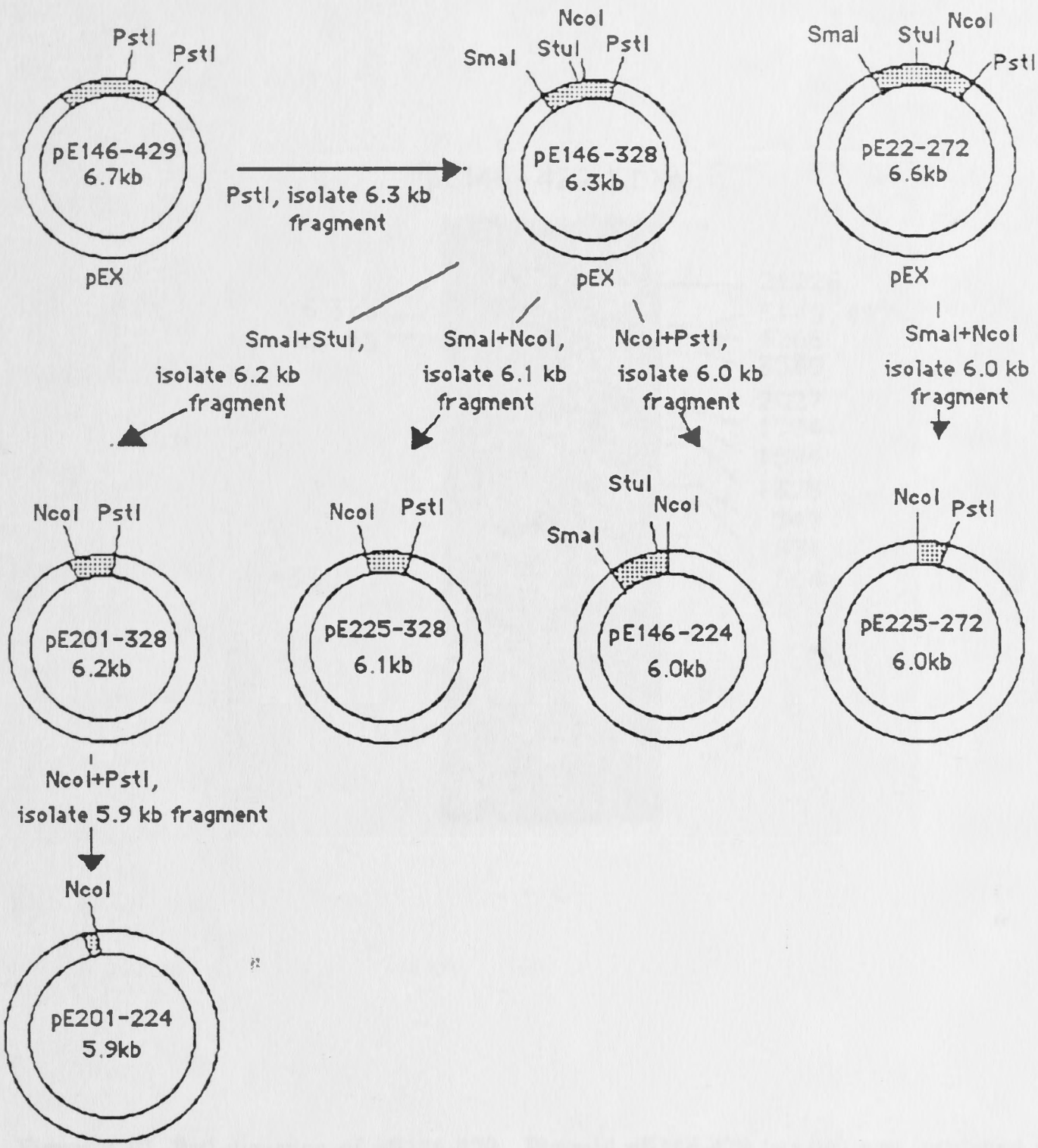
pE201-328: pE146-328 (1 μg) was incubated with SmaI (2 units) at 25° for 1 hour and then StuI (2 units) at 37° for 1 hour. The fragments (already blunt-ended) were separated on 1% LMT agarose; two fragments were observed (6.2 kb and <200 bp). The larger fragment was self-ligated and transformed into *E.coli* as above.

pE225-328: pE146-328 (1 μg) was incubated with SmaI (2 units) at 25° for 1 hour and then NcoI (2 units) at 37° for 1 hour and incubated with T4 DNA polymerase (0.2 units) in the presence of dNTPs (0.5 mM each at 37° for 30 min). The blunt-ended fragments were separated on 1% LMT agarose; two fragments were observed (6.1 kb and ≈200 bp). The larger fragment was self-ligated and transformed into *E.coli* as above.

pE146-224: pE146-328 (1 μg) was incubated with NcoI and PstI (5 units each) at 37° for 1 hour and incubated with T4 DNA polymerase (as above). The blunt-ended fragments were separated on 1% LMT agarose; two fragments were observed (6.0 kb and ≈300 bp). The larger fragment was self-ligated and transformed into *E.coli* as above.

pE225-272: pE22-272 (1 μg) was incubated with SmaI and NcoI (5 units each) and incubated with T4 DNA polymerase (as above). The blunt-ended fragments were separated on 1% LMT agarose; two fragments were observed (6.0 kb and ≈600 bp). The larger fragment was self-ligated and transformed into *E.coli* as above.

pE201-224: pE201-328 (1 μg) was incubated with NcoI and PstI (5 units each) at 37° for 1 hour and incubated with T4 DNA polymerase (as above). The blunt-ended fragments were separated on 1% LMT agarose; two fragments were observed (5.9 kb and ≈300 bp). The larger fragment was self-ligated and transformed into *E.coli* as above.


PstI
PstI
pE146-429
6.7kb
pEX
PstI, isolate 6.3 kb fragment
NcoI
SmaI
StuI
PstI
pE146-328
6.3kb
pEX
SmaI
StuI
NcoI
PstI
pE22-272
6.6kb
pEX
SmaI+StuI, isolate 6.2 kb fragment
SmaI+NcoI, isolate 6.1 kb fragment
NcoI+PstI, isolate 6.0 kb fragment
SmaI+NcoI isolate 6.0 kb fragment
NcoI PstI
pE201-328
6.2kb
NcoI+PstI, isolate 5.9 kb fragment
NcoI
pE201-224
5.9kb
NcoI PstI
pE225-328
6.1kb
StuI
NcoI
SmaI
pE146-224
6.0kb
NcoI PstI
pE225-272
6.0kb

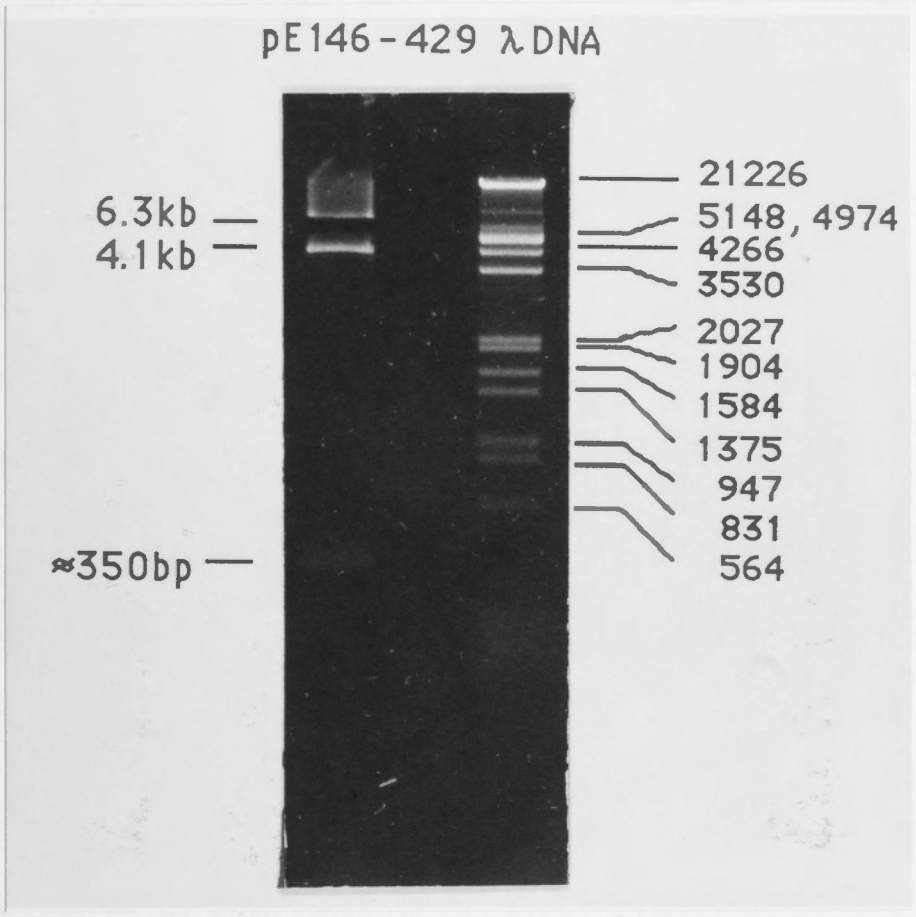


Figure 3.10 PstI-digestion of pE146-429. Plasmid pE146-429 (≈3 μg) was incubated with PstI (5 units) at 37° for 1 hour and then 70° for 10 min. Restriction fragments were electrophoresed on 1.5% LMT agarose at 60 volts for 1 hour. The agaros gel was stained in ethidium bromide (0.5 μg/ml) for 30 min and viewed on a UV-transilluminator. A band corresponding to ≈350 bp was excised from the gel for cloning into pEX vectors.

The MVE insert in pE327-429 was labelled at the 3' end with ^{32}P-dCTP and sequenced by the chemical method (Materials and Methods; Chapter 2). The sequence data obtained confirmed the identity of pE327-429. Plasmids pE201-328, pE225-328 and pE225-272 were partially sequenced between the polylinker and E insert to ensure that translation of the E insert was in frame with ß-gal*.

Molecular weight and yield of MVE E fusion proteins encoded by recombinant plasmids

The mol. wts. of the fusion proteins were predicted from the lengths of the lacZ*-E fused gene (Table 3.2). Fusion proteins from lysates were analysed by SDS-PAGE for comparison with these predicted sizes to determine whether the expected translation had occurred. The results for the seven deleted recombinant pEX plasmids (Fig. 3.8) are shown in Table 3.2. The observed mol. wts. corresponded with the predicted mol. wts. and the coding capacity of inserts in recombinant plasmids. This indicated correct translation and absence of degradation of fusion proteins. There was no significant difference in yields between different fusion proteins and approximately 40 mg of each fusion protein per litre of induced culture was obtained (Fig. 3.3).

Reactivity of anti-E-8 mAb with MVE E fusion proteins

The seven fusion proteins were used to map the E-8 epitope. To test for reactivity with anti-E-8, approximately equimolar quantities of fusion proteins were used. Bacterial lysates were reduced, electrophoresed on SDS-PA gels and nitrocellulose blots analysed using anti-E-8 mAb. Anti-E-8 reacted with E146-328 but not with E327-429 (Fig. 3.7) eliminating the latter sequence as a significant part of the epitope. There was no reduction in reactivity of E146-328 relative to E22-272 and E146-429. Thus it appeared that the binding site remained intact in E146-328. Three fusion proteins, E201-328, E146-224 and E201-224 from within this region also reacted with anti-E-8 (Fig. 3.7). E201-328 showed reactivity similar to E146-328. E146-224 and E201-224 showed weaker reactivity than E146-328 (by approximately 50%).

TABLE 3.2

Predicted and observed mol. wts. of MVE fusion proteins

Fusion protein	Predicted mol. wt.[a] (K)	Observed mol. wt.[b] (K)
E22-272 (Asp 22-Val 272)	149 (251)	156
E146-429 (Ser 146-Gly 429)	153 (285)	158
E146-328 (Ser 146-Gln 328)	142 (184)	145
E327-429 (Leu 327-Gly 429)	133 (103)	139
E146-224 (Ser 146-Pro 224)	131 (80)	135
E201-328 (Tyr 201-Gln 328)	136 (128)	142
E201-224 (Tyr 201-Pro 224)	125 (24)	124
E225-328 (Trp 225-Gln 328)	133 (104)	139
E225-272 (Trp 225-Val 272)	127 (48)	131

[a] Calculated from the observed mol. wt. of ß-gal* (122K; see Chapter 2) and additional amino acids from the E protein of MVE (Dalgarno *et al.*, 1986). The predicted numbers of amino acids are shown in brackets.

[b] Determined by SDS-PAGE (Fig. 3.3).

These observations suggested that the major binding site of anti-E-8 was located within a stretch of 24 amino acids between Tyr 201 and Pro 224 in the MVE E protein. Since fusion proteins lacking $E_{225-328}$ showed decreased reactivity, this region may also influence the binding of anti-E-8. Since this mAb did not react with fusion proteins E225-272 or E225-328 in immunoblots, this influence is presumably indirect.

A search was made of restriction sites located within the region coding for peptide $E_{201-224}$ for further deletion mapping. No appropriate sites were available. Thus alternative methods would have to be employed to identify the critical residues within E201-224 which were essential for binding of anti-E-8.

3.3.4 Reactivity of anti-MVE HIAF with deleted fusion proteins

To assess the suitability of the fusion proteins for use in immunization experiments (see below), they were examined for the presence of MVE epitopes using polyclonal HIAF. Lysates of bacterial cells expressing the nine available fusion proteins were electrophoresed, after reduction, on an SDS-7.5% PA gel and immunoblotted using anti-MVE HIAF. Approximately equimolar amounts of fusion proteins were analysed. In each bacterial lysate, a single antibody-reactive band was observed (see Fig. 3.4), corresponding in size with the fusion protein band observed in Coomassie Blue stained profiles (Fig. 3.3). Consistent differences in the reactivities of these reduced fusion proteins were observed in several experiments. E201-224 was only detected using more concentrated HIAF and large quantities of protein (results not shown). The weak reactivity of E201-224 may be due to the small size of the E peptide (24 amino acids). Differences in immunoreactivities of other proteins may have been influenced by the number of epitopes in these proteins or the resistance of the epitopes to reduction and denaturation. The strong immunoreactivity of E225-272 relative to E225-328 was unexpected because MVE peptide $E_{225-272}$ is also present in fusion protein E225-328. This may indicate that in the latter fusion protein, the reactivity of epitopes between

Trp 225 and Val 272 was adversely affected by the presence of sequences between Glu 273 and Gln 328.

These results suggested that each of the E fusion proteins contained reactive MVE antigenic sites. There were however differences in the overall reactivities with HIAF. Thus it was of interest to determine whether these fusion proteins elicited antibodies against MVE in mice.

3.3.5 Isolation of fusion proteins for mouse injection

Fusion proteins for injection of mice were partially purified as follows. *E.coli* cultures were induced for fusion protein synthesis and cells harvested after two hours. Lysates containing ß-gal*, E22-272, E146-429 or E201-224 were prepared by freeze-thawing and lysozyme treatment in the absence of detergent to avoid irreversible denaturation, solubilization of fusion proteins and problems of detergent removal. The fusion proteins were insoluble under these conditions and were pelleted by centrifugation; they were extracted and solubilized with 8 M urea which was then removed by exhaustive dialysis against PBS. The yield ranged from 40-50 mg/litre of induced culture. As the fusion protein content of total bacterial lysates was approximately 40 mg/litre (see above), very little loss had occurred during the procedure. Approximately 95% of the stained protein in the preparations was fusion protein (Fig. 3.11A), by comparison with approximately 80% in induced bacterial lysates. Fusion proteins appeared as doublets after partial purification. This may indicate partial degradation or incomplete renaturation after urea treatment.

The possibility that the conformation of fusion proteins was altered during purification was examined by assessing their immunoreactivities with HIAF and anti-E-8 mAb. Purified fusion proteins were dot-blotted on nitrocellulose or electrophoresed in the reduced state and transferred to nitrocellulose for immunodetection. After purification, E22-272, E146-429 and E201-224 remained reactive to both HIAF and

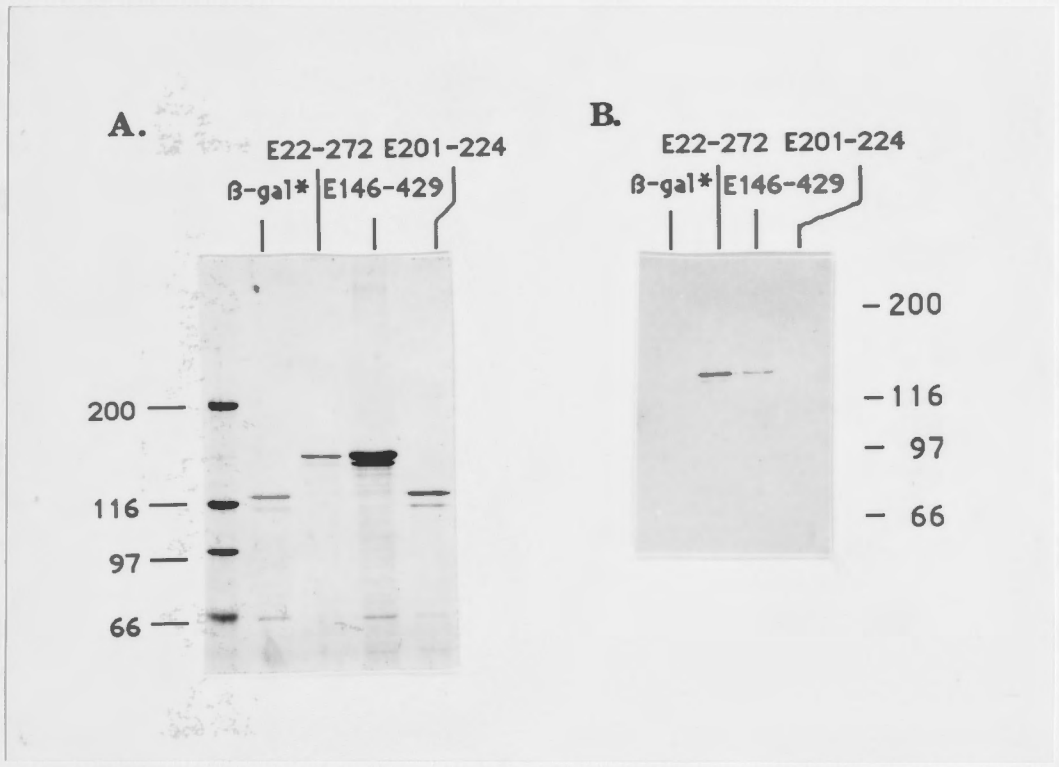


Figure 3.11 SDS-PAGE profiles and immunoreactivity of purified fusion proteins. (A) SDS-PAGE profiles of purified fusion proteins. ß-gal* and fusion proteins E22-272, E146-429 and E201-224 were purified from bacterial lysates by extracting the insoluble fractions. The purified proteins (0.5-1 µg each) were incubated with ß-ME at 90° for 2 min and electrophoresed on an SDS-7.5% PA gel at 200 volts for 45 min. Proteins were stained with Coomassie Brilliant Blue. The size markers were as described in Fig. 3.3.

(B) Immunoblot analysis of purified fusion proteins. ß-gal* and fusion proteins E22-272, E146-429 and E201-224 were separated by SDS-PAGE as above and transferred to nitrocellulose for detection using anti-MVE HIAF as in Fig. 3.4.

anti-E-8 (Fig. 3.11B; results for anti-E-8 not shown)*. Doublets were detected on immunoblots indicating that both 'species' were antigenic.

3.3.6 Injection of mice with fusion proteins in Freund's incomplete adjuvant

ELISA titres against fusion proteins

Outbred Swiss mice at 6-8 weeks of age were injected with three fusion protein preparations (E22-272, E146-429 and E201-224; 10 µg per mouse) emulsified in Freund's incomplete adjuvant (FIA). Booster injections with identical preparations were given four and nine weeks after the initial injection. Groups of five mice were injected with each fusion protein. ß-gal*, prepared identically to fusion proteins, was injected into a control group of mice. Antibody titres against immunizing proteins were determined by ELISA (Table 3.3). Reactivity was detected in most mice 14 days after injection but titres were low. Titres generally increased after each boost; after two boosts, they ranged from 200 to 12,800. Differences were observed between animals injected with identical fusion proteins (Table 3.3). ELISA titres against fusion proteins were generally highest for E201-224, intermediate for E146-429 and lowest for E22-272. When ELISA titres against ß-gal* were tested after a single boost, it was found that there was little difference between anti-ß-gal* and anti-fusion protein reactivities (Table 3.3). Thus most of the immune response was directed at the ß-gal* sequence which made up 78-97% of the immunizing fusion proteins.

ELISA titres against MVE

To determine whether any of the above antisera were reactive against virus, ELISA was performed with partially purified MVE as antigen. The results (Table 3.3) showed that anti-MVE reactivities were detected for two out of 14 mice. One mouse injected with E22-272 (no. 3) developed consistent anti-MVE ELISA titres (320, 800) after two boosts; the second mouse, also injected with E22-272 (no. 4), was positive at 11 weeks

* In Fig. 3.11B, the detection of E201-224 by anti-MVE HIAF was not effective owing to insufficient quantity of fusion protein (see Section 3.3.4).

TABLE 3.3

Anti-fusion protein and anti-MVE ELISA titres of sera from mice injected with fusion proteins emulsified in Freund's incomplete adjuvant

Fusion protein	Mouse	ELISA titres[a] Weeks post injection						N titres
		0	2	5	6	10	11	
E22-272	1	-[b](-)	25(-)	ND[c](-)	100(-)[d]	200(-)	400(-)	-[b]
	2	-(-)	25(-)	ND(-)	100(-)	400(-)	800(-)	-
	3	-(-)	25(-)	25(-)	100(-)[e]	400(320)	800(800)	-
	4	-(-)	-(-)	ND(-)	-(-)	200(-)	800(25)	-
	5	-(-)	10(-)	ND(-)	100(-)	400(-)	400(-)	-
E146-429	1	-(-)	10(-)	1600(-)	1600(-)	3200(-)	3200(-)	-
	2	-(-)	10(-)	400(-)	400(-)	3200(-)	3200(-)	-
	3	-(-)	25(-)	800(-)	3200(-)[f]	800(-)	1600(-)	-
	4	-(-)	25(-)	200(-)	800(-)	3200(-)	3200(-)	-
E201-224	1	-(-)	10(-)	400(-)	ND(-)	1600(-)	ND(-)	-
	2	-(-)	-(-)	6400(-)	6400(-)	12800(-)	ND(-)	-
	3	-(-)	50(-)	3200(-)	6400(-)[g]	6400(-)	6400(-)	-
	4	-(-)	25(-)	3200(-)	6400(-)	12800(-)	ND(-)	-
	5	-(-)	10(-)	3200(-)	12800(-)	12800(-)	ND(-)	-

[a] Anti-fusion protein ELISA titres are given first; figures in brackets represent anti-MVE ELISA titres.
[b] Titres lower than 10 are represented as -.
[c] Not done.
[d] Anti-ß-gal* titre was 200.
[e] Anti-ß-gal* titre was 100.
[f] Anti-ß-gal* titre was 3200.
[g] Anti-ß-gal* titre was 6400.

after injection but gave a much lower titre. E201-224 and E146-429 failed to elicit detectable anti-MVE responses in mice.

3.3.7 Immunization with fusion proteins in RIBI adjuvant

Two factors may have contributed to the failure of certain E fusion proteins to elicit anti-MVE antibodies: the E components may not have been strongly immunogenic or the conformation may not have been native. An attempt was made to improve the immunogenicity of E peptides in fusion proteins. First, FIA was replaced with RIBI adjuvant (see Materials and Methods) which is reported by the manufacturer to elicit high antibody titres and require fewer booster injections by comparison with FIA and Freund's complete adjuvant. RIBI has been used successfully to elicit anti-MVE antibodies with synthetic peptides (Roehrig *et al.*, 1989). Second, the fusion proteins were altered by removing a large portion of the ß-galactosidase sequence to increase the proportion of MVE in fusion proteins.

Generation of fusion proteins containing truncated ß-galactosidase

Truncation of the ß-galactosidase sequence was achieved by removing a portion of the coding region at the 3' terminus. The aim was to express fusion proteins containing a smaller ß-galactosidase fragment without altering the polylinker sequence. The existence of a sequence (1.9 kb) which encompassed 63% of the lacZ* coding region and is flanked by the unique EcoRV and EcoRI sites in pEX2 (Fig. 2.1) allowed the following strategy to be adopted. Incubation of pEX2 with EcoRV and EcoRI generated two fragments (3.9 kb, 1.9 kb). The larger one was the linearized pEX2 plasmid containing the truncated lacZ coding region. The polylinker region and the essential features of the pEX plasmid for expression of foreign genes were intact. The 3.9 kb fragment was isolated (Fig. 3.12), blunt-ended with T4 DNA polymerase and self-ligated. After transformation, ampicillin resistant clones were selected, plasmids extracted and analysed by PstI digestion and gel electrophoresis. Plasmids were 3.9 kb in size and contained the PstI site in the polylinker region consistent with the desired

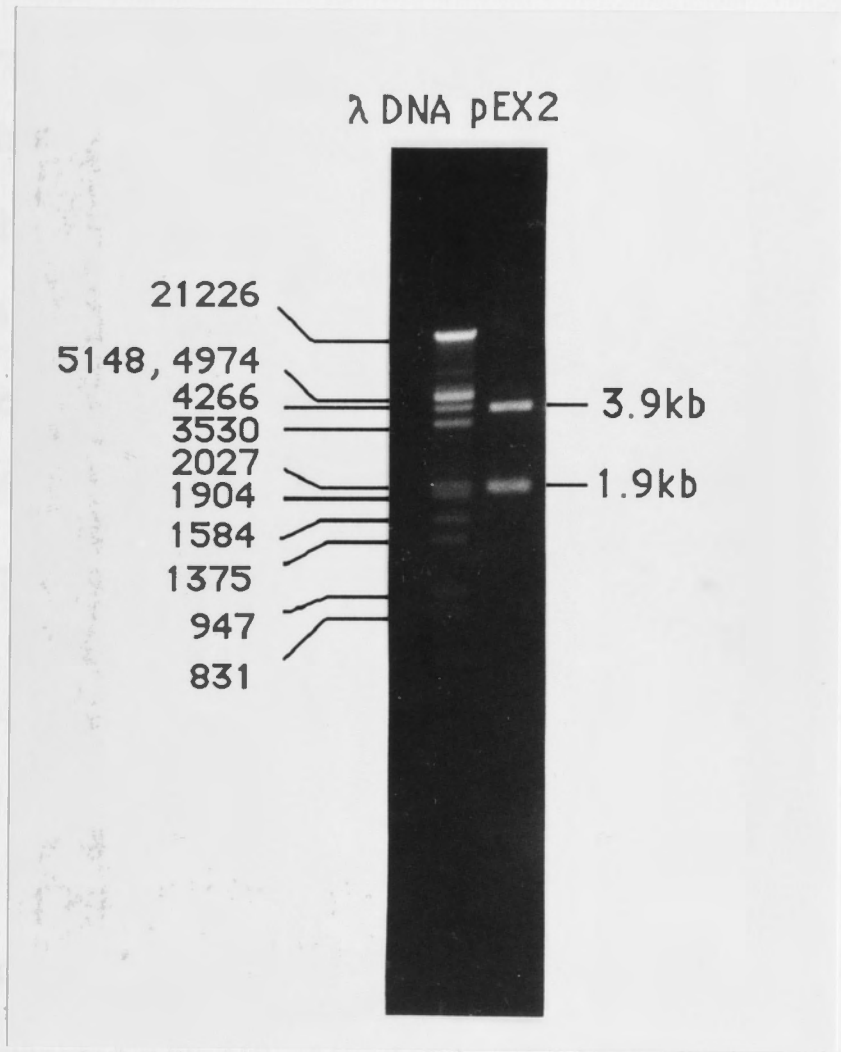


Figure 3.12 Construction of pEX2D. pEX2 plasmid (1 μg) was incubated with EcoRV and EcoRI (5 units each) at 37° for 1 hour and then with T4 DNA polymerase (0.2 units) in the presence of dNTPs (0.5 mM each). The digest was electrophoresed on 1% LMT agarose, soaked in ethidium bromide (0.5 mg/ml) for 30 min and viewed on a UV-transilluminator. The larger fragment (3.9 kb) was excised from the gel for self-ligation to generate pEX2D.

plasmid which was designated pEX2D (Fig. 3.13A). The ß-galactosidase sequence encoded in this plasmid was predicted to be 375 amino acids long and is referred to below as ß-galD.

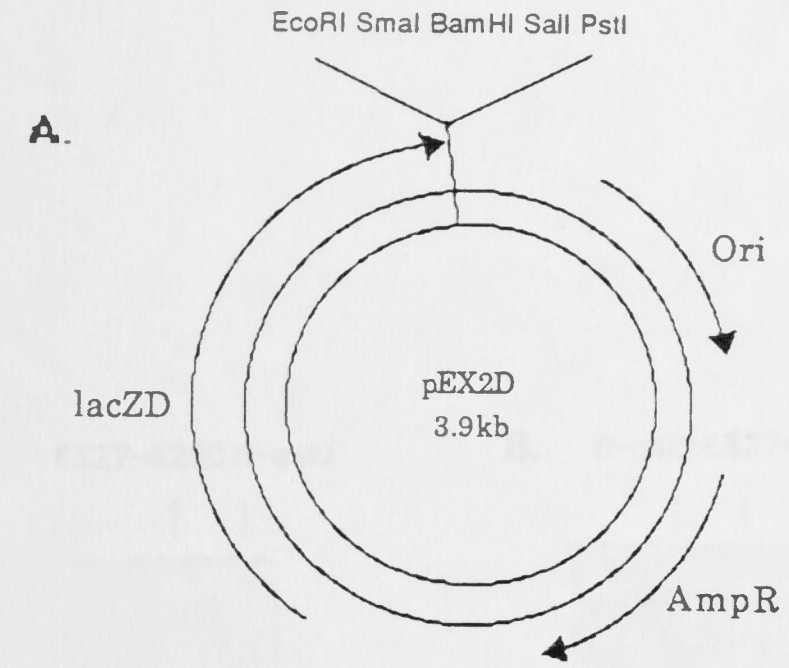
pEX2D was ligated with a cDNA fragment encoding $E_{327-429}$ to generate a plasmid designated pE327-429D. The cDNA (≈350 bp) was derived from pE146-429 by PstI digestion as for the construction of pE327-429 (Fig. 3.10). It was ligated to PstI-digested, dephosphorylated pEX2D. Transformed ampicillin resistant colonies were screened with HIAF. A strongly positive clone was picked and the plasmid extracted and analysed by gel electrophoresis after PstI digestion. It contained the two expected PstI fragments of 3.9 kb and ≈350 bp (results not shown), consistent with the restriction sites in pE327-429D and with its size (Fig. 3.13B).

Fusion proteins were prepared from expression of pEX2D and pE327-429D. Approximately 50 mg ß-galD (52K) and E327-429D (63K) were obtained per litre of culture. The sizes of ß-galD and E327-429D (see Fig. 3.14A) were consistent with the presence of 103 amino acids from the MVE E protein in E327-429D. We conclude that E327-429D was extracted without degradation in a yield similar to that for ß-gal* fusion proteins.

The reactivity of E327-429D to HIAF was examined after dot-blotting on nitrocellulose (results not shown) or after SDS-PAGE and immunoblotting (Fig. 3.14B). E327-429D reacted strongly in both assays and was therefore a suitable replacement for E327-429 in immunization experiments.

ELISA titres against fusion proteins

Preparations of ß-galD, E327-429D and E201-224 were injected into mice with RIBI adjuvant. The dose of each was 10 µg/mouse. Because of the cost of the adjuvant, the number of mice injected was reduced to two per fusion protein. Serum samples were collected before and 14 days after injection. A booster injection with an



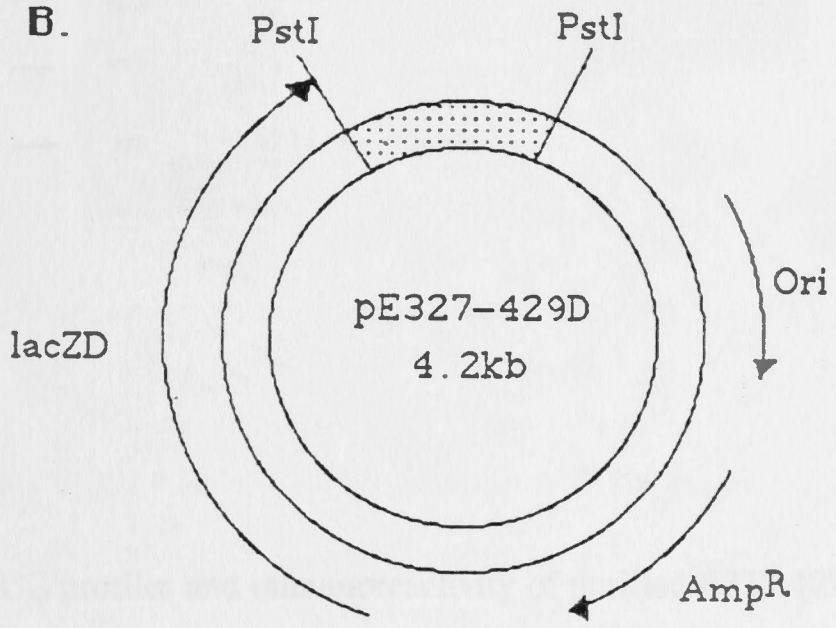


Figure 3.13 pEX2D and pE327-429D plasmids. (A) Plasmid pEX2D (3.9 kb) was created by truncating the lacZ* gene (3 kb) in pEX2 (Fig. 2.1) to lacZD (1.1 kb) leaving the 5' portion intact. (B) Plasmid pE327-429D (4.2 kb) comprises pEX2D and the E gene fragment coding for the MVE peptide $E_{327\text{-}429D}$.

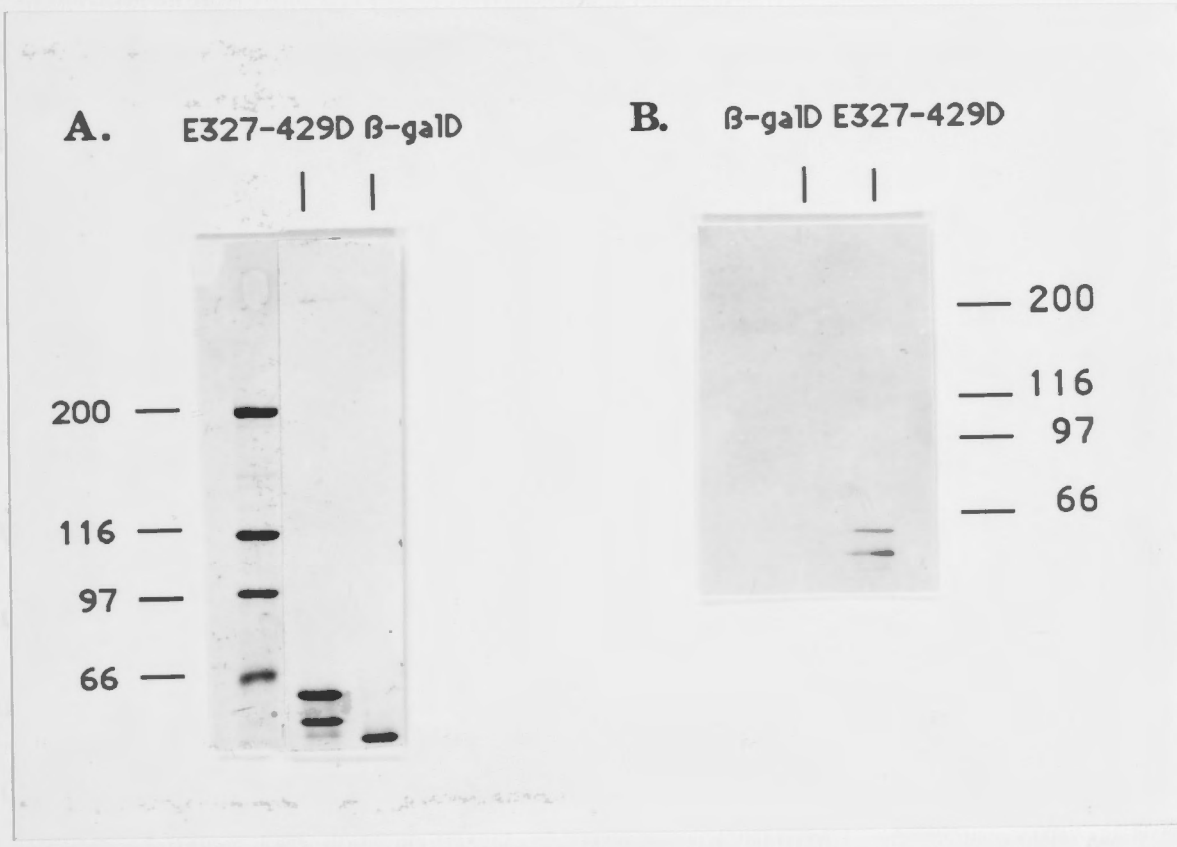


Figure 3.14 SDS-PAGE profiles and immunoreactivity of purified E327-429D and ß-galD. (A) SDS-PAGE profiles of purified E327-429D and ß-galD. ß-galD and fusion protein E327-429D were purified from bacterial lysates by extracting the insoluble fractions. The purified proteins (1 µg each) were incubated with ß-ME at 90° for 2 min and electrophoresed on an SDS-7.5% PA gel. Proteins were then stained with Coomassie Brilliant Blue. The size markers were as described in Fig. 3.3.
(B) Immunoreactivity of fusion protein E327-429D. Purified preparations of E327-429D and ß-galD (1 µg each) were electrophoresed on an SDS-7.5% PA gel and transferred to nitrocellulose. Detection was with anti-MVE HIAF (1 in 800 dilution; 1 hour incubation).

identical preparation was given five weeks after the initial injection; serum samples were collected seven and 14 days after boost.

The response was determined by ELISA in microtitre trays coated with E201-224 or E327-429D (Table 3.4). ELISA titres of 50-800 were observed two weeks after injection with the two fusion proteins in RIBI. The ELISA titres after the boost at five weeks were 25,600-51,200. In the earlier experiment (Table 3.3), ELISA titres no greater than 12,800 were obtained for mice injected with E201-224 in FIA. This represented a large (2 to 16-fold) increase in ELISA titres which may be related to the adjuvant used. However, this cannot be stated with certainty due to the small sample number.

The reactivity of sera to sequences in ß-galD was assessed by ELISA in microtitre trays coated with ß-galD. ELISA titres against ß-galD were approximately 4-fold lower than against E327-429D (Table 3.4; legend). This indicated that the sera contained antibodies directed at the MVE component.

ELISA titres against MVE

The reactivity of these sera with MVE was examined by ELISA. Both E327-429D and E201-224 elicited anti-MVE antibodies, detectable two weeks after injection. The titres after one boost rose to 200-400 for both E201-224 and E327-429D (Table 3.4). We conclude that the E peptides in these fusion proteins are immunogenic and elicit antibodies which react with epitopes in MVE.

3.3.8 Neutralization activity of anti-fusion protein sera

It was of interest to determine whether the sera which reacted with MVE in an ELISA neutralized virus infectivity. The question of whether E201-224 elicited neutralizing antibodies was of particular interest because this region of E appeared to define the E-8 neutralization epitope. Neutralization assays (PRNT) were carried out with sera from mice injected with E22-272, E201-224 and E327-429D and with control

TABLE 3.4

Anti-fusion protein and anti-MVE ELISA titres of sera from mice injected with fusion proteins in RIBI adjuvant

Protein	Mouse	ELISA titre[a] Weeks post injection 0	2	6	7	N titres
E201-224	1	-[b] (-)	800 (-)	25,600 (200)	ND[c] (200)	-[b]
	2	- (-)	50 (-)	51,200 (400)	ND (400)	10
E327-429D	1	- (-)	800 (-)	51,200 (200)[d]	ND (100)	10
	2	- (-)	400 (10)	204,800 (400)[e]	ND (200)	10

[a] Determined against immunizing fusion proteins. Figures in brackets represent anti-MVE titres.

[b] Titres lower than 10 are represented as -.

[c] Not done.

[d] Anti-ß-galD titre was 12,800.

[e] Anti-ß-galD titre was 51,200.

sera (Table 3.4). The anti-MVE ELISA titres of these sera ranged from 100-800. Sera from mice injected with E22-272 in FIA did not neutralize MVE infectivity. Similarly, sera from mice injected with fusion proteins E327-429D and E201-224 in RIBI adjuvant did not neutralize to a significant degree.

3.3.9 Summary

For epitope mapping on E fusion proteins, a recombinant pEX plasmid which expressed the N-terminal half of the MVE E protein (fusion protein E22-272) was first generated. The reactivity of five neutralizing mAbs with E22-272 and E146-429 (Chapter 2) was examined to assess the possibility of mapping the corresponding epitopes by deletion analysis. All five mAbs reacted specifically with unreduced MVE E protein in immunoblots. They differed in reactivity with the reduced protein. Anti-E-8 reacted with reduced E, though to a decreased level than with unreduced E. The reactivities of anti-E-1c, E-1d, E-5b and E-7 mAbs with MVE E protein were abolished by reduction of disulfide bonds. Anti-E-8 mAb reacted with both E22-272 and E146-429 fusion proteins. Epitope mapping by deletion analysis showed that residues 201-224 were essential for the interaction of the anti-E-8 mAb with a number of fusion proteins. The mAbs against E-1c, E-1d, E-5b and E-7 did not react with either unreduced or reduced E fusion proteins.

Mice were injected with partially purified fusion proteins to generate antisera for tests of reactivities against MVE in ELISA and neutralization assays. Four fusion proteins, E22-272, E146-429, E201-224 and E327-429D (the latter contained 40% of the ß-galactosidase component of other fusion proteins) in either FIA or RIBI adjuvant, were used. All four fusion proteins elicited antibodies against themselves. E22-272 in FIA, and E201-224 and E327-429D in RIBI elicited antibodies against MVE. None of the mouse sera neutralized MVE infectivity to a significant degree.

3.4 DISCUSSION

The aim of the work described in this chapter has been to define neutralization epitopes in the MVE E protein using ß-gal*-E fusion proteins generated in *E.coli*. In one approach we have subjected the E gene to a series of deletions, characterized the resulting fusion proteins and examined their reactivities with neutralizing mAbs to map neutralization epitopes. In the second approach, the capacity of mouse sera generated against fusion proteins to recognize MVE in ELISA and to neutralize MVE infectivity in plaque assays was assessed.

Reactivities of fusion proteins with neutralizing mAbs

From immunoblotting experiments using MVE electrophoresed on SDS-PAGE, it was clear that all five neutralizing mAbs reacted with epitopes in the authentic viral envelope protein and that these epitopes were either resistant to SDS denaturation or renatured after SDS denaturation. On the other hand, four mAbs (all except anti-E-8) failed to react with reduced E. We concluded that these four epitopes are likely to reside in domains of E which are created by or stabilized by disulfide bonds. By analogy with the WN and TBE E proteins (Nowak and Wengler, 1987; Mandl *et al.*, 1989b) they could reside in any region of the E protein, with the exception of domain C (residues 150-170) and the hydrophobic C-terminus (residues 460-501). We could not obtain further information on E-1c, E-1d, E-5b and E-7 epitopes as the corresponding mAbs did not react with either the unreduced or reduced E fusion proteins, indicating that these epitopes are absent in the E fusion proteins. Possible explanations are that these epitopes were not generated in the E fusion proteins owing to a distorted conformation, or to the need for the association of discontinuous stretches of the E components, or that the epitopes were located in parts of E not represented in the fusion proteins (residues 1-21 and residues 429-501). The epitope mapping studies on JE E-fusion proteins (Mason *et al.*, 1987b; Mason *et al.*, 1989) are of interest as they provide a direct comparison using a similar strategy. Of the eleven mAbs examined (these differ in degrees of cross-reaction with different flaviviruses and in activities in passive

protection), ten which neutralized JE infectivity reacted with JE fusion proteins containing the region in E between Met 303 and Trp 396. This indicated that the domain in JE E containing these epitopes, which corresponds to TBE domain B (see Fig. 1.4) can retain reactivity when expressed as fusion proteins (with trpE or ß-galactosidase) in *E.coli*. The reactivity of these mAbs appeared to require disulfide bonds as there was no reaction with reduced and carboxymethylated E or with fusion proteins lacking the cysteine in E at 304 (which forms disulfide with Cys 335). The MVE mAbs against E-1c, E-1d, E-5b and E-7 were not reactive with unreduced E146-429. As E146-429 contains a similar domain B, it seems likely that these epitopes are located in parts of MVE E protein outside the region Met 304-Trp 397. In summary, our data suggest that these epitopes are stabilized by disulfide bond formation and the overall conformation of E, and that they are not in a domain corresponding to TBE domain B.

Anti-E-8 reacted with the two largest fusion proteins E22-272 and E146-429, showing approximately equivalent reactivities on a molar basis with MVE E protein. Approximately half of this reactivity was associated with a small section of E (Tyr 201-Pro 224) which corresponds to part of domain A. Four TBE neutralization epitopes have been demonstrated in domain A; two of them, the A5 determinant (Glu 207) and the A4 determinant (Gln 233) map close to the E-8 region defined here (Mandl *et al.*, 1989b). A point of interest was the degree to which the reactivity of the E-8 antigenic site was determined by its 'environment' in E. E22-272, E146-429 and E146-328, which all carried the peptide $E_{201-224}$, had approximately equivalent reactivities on a molar basis. On the other hand, E146-224 and E201-224 showed a fall (of ≈50%) in reactivity compared with fusion proteins also containing $E_{225-328}$. From this we conclude that sequences between Trp 225 and Gln 328 are required for full reactivity with anti-E-8, but are not reactive alone (see below).

MAb against the E-8 epitope (Table 3.1) cross-reacts in ELISA with JE, SLE and WN and also in protection asssays in mice with JE and SLE. Fig. 3.15 shows the sequence of the MVE E protein from Tyr 201 to Pro 224 aligned with that of other flaviviruses. Fig. 3.16 (adapted from Dalgarno *et al.*, 1986) shows the hydropathy characteristics of the E-8 region and its surrounds. The sequence contains two hydrophobic regions (at the N- and C-termini) and a hydrophilic central region (underlined in Fig. 3.15). The sequence homology in the region 201-224 between MVE and JE, WN or SLE is 75, 75 and 58% respectively. Homology is considerably higher at the C-terminal end than overall. Since cross-reaction occurs with SLE (which shows ≈70% homology in the C-terminal 13 amino acids comprising the hydrophilic segment, and only ≈45% in the N-terminal section), it seems likely that the epitope recognized by anti-E-8 mAb is in fact in the C-terminal half (residues 212-224).

Finally, it is of interest that although a disulfide bond presumably links MVE residues 190 and 288, and the E-8 epitope falls within this loop, reactivity was lowered but not abolished by ß-mercaptoethanol treatment. This suggests that within a domain which is stabilized by disulfide bonds, significant variations exist in sensitivity to reduction or that disulfide bond reformation has occurred. MAbs against epitopes in domain A of TBE lose reactivity after E is reduced and carboxymethylated, a treatment which disrupts disulfide bonds irreversibly (Guirakhoo *et al.*, 1989). As reduction of MVE was achieved by ß-ME treatment alone, we cannot rule out the possibility that renaturation occurred.

Conformation of viral proteins expressed in *E.coli*

The lack of reactivity of the neutralizing mAbs against E-1c, E-1d, E-5b and E-7 epitopes with E22-272 and E146-429 and the inability of fusion proteins to elicit

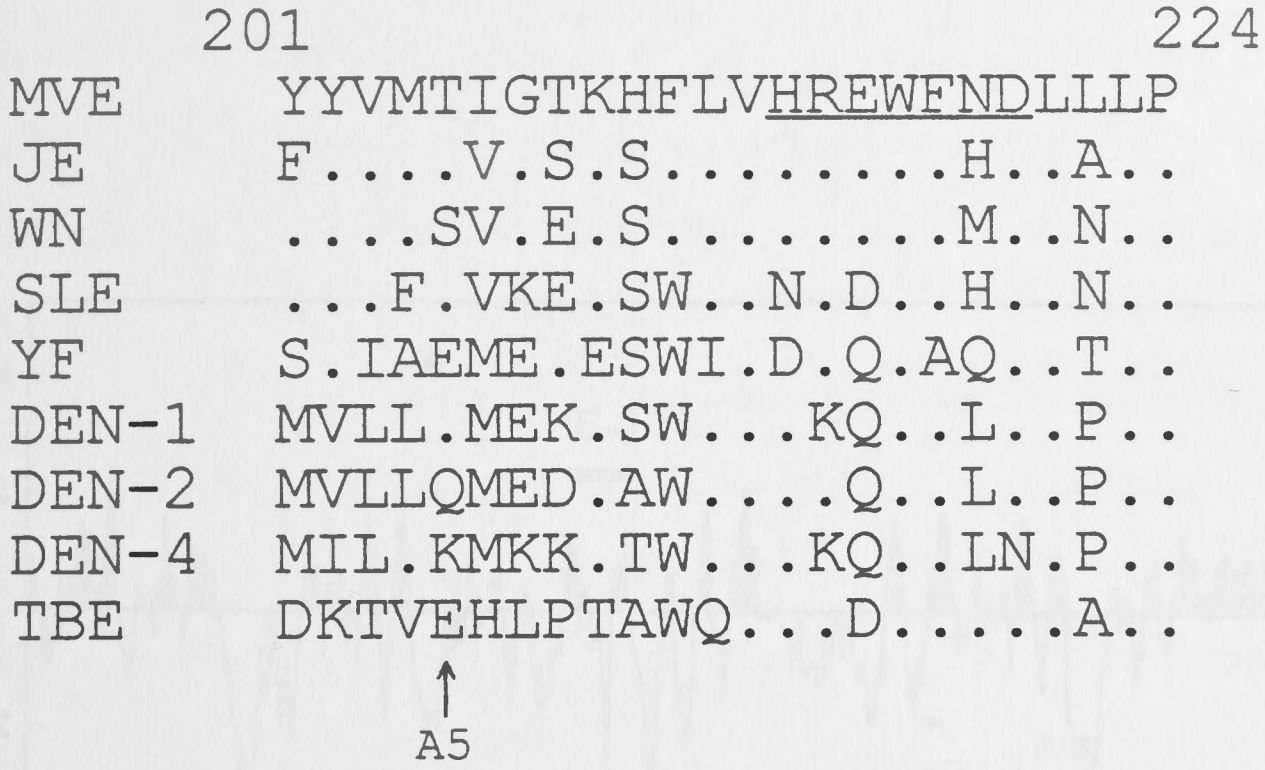


Figure 3.15 Amino acid sequence of the MVE E protein from Tyr 201 to Pro 224. The MVE sequence is shown on the top line with an underlined segment representing a strongly hydrophilic region. Aligned sequences below are for JE (McAda *et al.*, 1987), WN (Castle *et al.*, 1986), SLE (Trent *et al.*, 1987), YF 17D (Rice *et al.*, 1985), DEN-1 (Mason *et al.*, 1987a), DEN-2 (Deubel *et al.*, 1986), DEN-4 (Zhao *et al.*, 1986) and TBE (Mandl *et al.*, 1988). The single letter amino acid code is used; dots represent amino acid residues identical to MVE at similar positions. The arrow indicates the A5 neutralization determinant of TBE (Mandl *et al.*, 1989b).

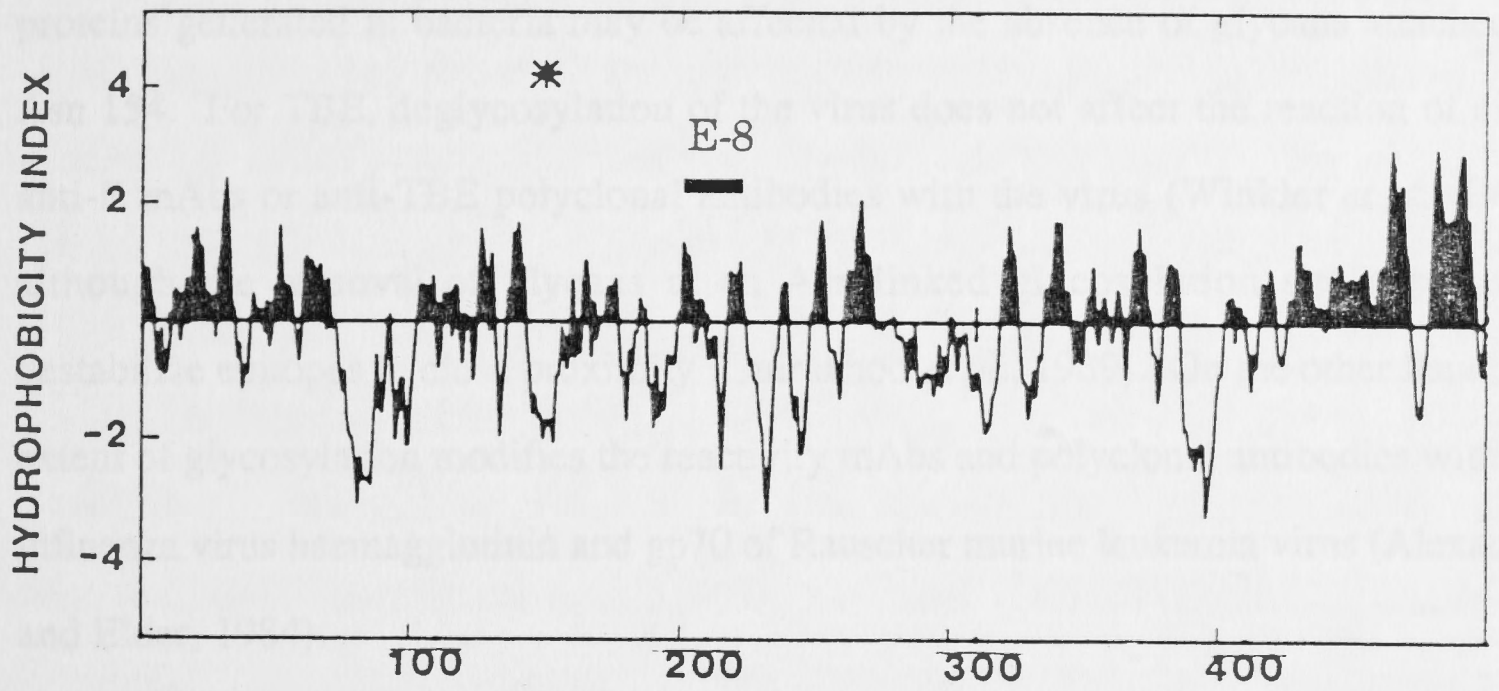


Figure 3.16 Hydropathy profile of the MVE E protein (adapted from Dalgarno *et al.*, 1986). The MVE E protein sequence (Dalgarno *et al.*, 1986) is subjected to hydropathy analysis (Kyte and Doolittle, 1982) and plotted on the vertical axis with positive values indicating hydrophobicity and negative values indicating hydrophilicity. The region from Tyr 201 to Pro 224 is indicated.

neutralizing antibodies in mice can be attributed to the conformation of the E polypeptide component of the fusion proteins. Three factors may be involved in this. First, MVE E protein has a conserved Asn-linked glycosylation site (at Asn 154) to which carbohydrates are normally attached (Winkler *et al.*, 1987). Residue 154 is present in both E22-272 and E146-429. As Asn-linked glycosylation only occurs in eukaryotic cells (Hubbard and Ivatt, 1981), the conformation of the E components of the fusion proteins generated in bacteria may be affected by the absence of glycans attached to Asn 154. For TBE, deglycosylation of the virus does not affect the reaction of eight anti-E mAbs or anti-TBE polyclonal antibodies with the virus (Winkler *et al.*, 1987) although the removal of glycans at an Asn-linked glycosylation site appears to destabilize epitopes in close proximity (Guirakhoo *et al.*, 1989). On the other hand, the extent of glycosylation modifies the reactivity mAbs and polyclonal antibodies with the influenza virus haemagglutinin and gp70 of Rauscher murine leukemia virus (Alexander and Elder, 1984).

The second factor is that bacterial proteins contain more sulfhydryl groups than eukaryotic proteins and this appears to be related to a stronger reducing environment in the bacterial cytosol (Fahey *et al.*, 1977). The MVE peptide $E_{22\text{-}272}$ would, as generated in eukaryotic cells, be expected to carry three disulfide bonds (Cys 60-121, Cys 74-105, Cys 92-116). Cys 30 and Cys 190, also present in E22-272, would form bridges with Cys 3 and Cys 288 respectively in the native protein (Nowak and Wengler, 1987). E146-429 would be expected to have two disulfide bonds (Cys 190-288 and Cys 305-336) but no cysteine residues without pairing possibilities. This protein would be more likely to form the correct conformation. In studies with JE, the presence of cysteines at residues 304 and 335 in the E protein was essential to the reactivity of mAbs with the fusion proteins used suggesting that the disulfide bond (Cys 304-335) in domain B was generated in the bacterial cytosol. Despite these indications, no confident predictions can be made concerning the conformation of $E_{22\text{-}272}$ and $E_{146\text{-}429}$ as part of fusion proteins in the *E.coli* cytosol.

A third consideration is that the fusion proteins used in this study carried no more than 284 amino acids of the MVE E protein fused with a ß-gal* protein of more than 1000 amino acids. The conformation of the MVE components may be influenced by interaction with the ß-galactosidase sequence. Thus epitopes which depend on specific folding of the E sequence or are assembled from distant regions of E may not acquire a conformation similar to that in native E protein.

The results from immunization experiments using the ß-gal-E fusion proteins were of relevance to our understanding of neutralization epitopes in MVE. We have shown that E201-224 in FIA, and E22-272 and E327-429D in RIBI adjuvant elicited antibodies which reacted with MVE. These antibodies did not neutralize MVE infectivity in plaque assays although a neutralizing mAb (anti-E-8) reacted with both E201-224 and E22-272. This indicated that the neutralization epitope in these fusion proteins did not elicit neutralizing antibodies in mice. As reactivity of E201-224 is only half that of E22-272, indicating incomplete or distorted presentation of the E-8 epitope, it is perhaps not surprising that this fusion protein cannot elicit neutralizing antibodies. Even though E22-272 may have the intact E-8 epitope, another factor may account for its inability to elicit neutralizing antibodies. Although the MVE component of E22-272 elicits a polyclonal reponse in mice, perhaps only a small proportion of antibodies are directed at the E-8 epitope. As the ELISA titre against MVE was not high (≈800), the magnitude of the anti-E-8 response may have been too small to neutralize infectivity.

In relation to flaviviruses in general, our work reinforces the view that conformation-dependent epitopes play a major role in neutralization. For JE, neutralization epitopes were mapped using fusion proteins to a region of E corresponding to TBE domain B (Mason *et al.*, 1989); however, these fusion proteins did not elicit neutralizing antibodies in mice. Thus the native and intact E may be required to elicit neutralizing antibodies in mice. For WN, E protein purified by SDS-PAGE elicits neutralizing antibodies in rabbits, while similar preparations of E reduced

by ß-ME do not (Wengler and Wengler, 1989b). For TBE, epitopes in both domains A and B are destroyed by irreversible reduction, and neutralization epitopes in domain A as well as the neutralization epitope i2 are also destroyed by SDS treatment (Guirakhoo *et al.*, 1989).

The application of E fusion proteins to studying the mechanism of virus entry and of neutralization of infectivity had been one goal of this work. However, these processes are likely to depend on the specific conformation of part or all of the E protein. In view of the results presented, it seems unlikely that MVE fusion proteins are sufficiently conformationally homologous to the native E protein to be useful in approaching these goals. The application of bacterial fusion proteins appears to be limited to mapping particular epitopes only and to generating specific antisera for use as protein-specific 'reagents'. To pursue our objective of studying the functional determinants of E, epitope mapping using mAb selected escape variants and the expression of E in a more 'native' conformation are explored in Chapters 4 and 5 respectively.

CHAPTER 4

SEQUENCE STUDIES AND VIRULENCE ASSAYS ON MVE ANTIGENIC VARIANTS

4.1 INTRODUCTION

In the first part of this chapter, we aim to map neutralization epitopes in the MVE E protein by sequencing mAb escape variants of MVE. As noted in Chapter 1, few data exist which define neutralization epitopes in members of the important JE serogroup of flaviviruses. Within this group which includes JE, MVE, SLE, WN and KUN, published data exist only for JE (Mason *et al.*, 1989). These investigators have shown that the region of E between residues 303 and 396 defines a domain against which ten neutralizing mAbs are directed; however, the residues recognized by each of the mAbs have not been defined. It has been shown for MVE that residue 126 is part of the epitope recognized by anti-E-1c mAb (S. H. Hartley and R. C. Weir, personal communication). For flaviviruses in general, the most extensive data available are for TBE; neutralization epitopes have been mapped to residues 67, 71, 171, 207, 233, 384 and 389 in the E protein (Mandl *et al.*, 1989b). For YF, neutralization epitopes have been mapped to residues 71 and 72 (Lobigs *et al.*, 1987); and for DEN-1 to residues 293-401 (Zuegel *et al.*, 1987). Due to the likely conservation of the basic architecture of the flavivirus E protein (Nowak and Wengler, 1987; Mandl *et al.*, 1989b), any data obtained on MVE antigenic sites could be of relevance to other flaviviruses, particularly to the medically important JE.

In the second part of this chapter, we describe virulence assays carried out with the sequenced MVE mAb escape variants. These studies were performed because of the possibility that in the flavivirus envelope protein, neutralization epitopes 'overlap' the region of E which recognizes a cell receptor molecule or a molecule which acts in virus cell interaction. Changes in such a region could modify virus-cell interaction, tissue tropisms and virulence. Of interest in this regard, a neutralization escape variant of TBE altered at a single residue in E (Tyr 384) is attenuated in mice (Heinz *et al.*, 1989). Furthermore, Lobigs *et al.* (1990) have shown that passage of MVE-1-51 in cell culture can lead to a specific change at Asp 390 in the E protein; this change from the wild-type

sequence is accompanied by attenuation of mouse virulence although a causal relationship has not been established.

4.2 MATERIALS AND METHODS

4.2.1 Viruses and cells

MVE-1-51 stocks used by Dr R. C. Weir and Dr J. T. Roehrig and for the work described in this study were derived from the same source (Yale Arbovirus Research Unit, USA). Working stocks of MVE-1-51 were tissue culture supernatants from infected Vero cell monolayers. MVE variant E-1c/V5b was provided by Dr R. C. Weir (see below).

Vero cells were grown in M199/LAH medium plus 10% bovine serum.

4.2.2 Monoclonal antibodies

Anti-E mAbs 4B6C-2, 4B5A-2, 4B3B-6, 6A4D-1 and 6B4A-10 which defined epitopes E-1c, E-1d, E-5b, E-7 and E-8 respectively (Table 3.1) were from Dr J. T. Roehrig. The first four were provided as both ascitic fluids and lyophilized ascitic fluids. The latter were reconstituted in sterile water upon arrival. Ascitic fluids were transported from USA at ambient temperature and were of low titre, presumably because of this; they were subsequently stored in aliquots at -70°. Freshly thawed aliquots of both forms of mAbs were used for neutralization tests.

4.2.3 Plaque reduction neutralization tests (PRNT)

Serial two-fold dilutions of antibody were in HBSS (pH 8). Diluted antibody (100 µl) was incubated with ≈200 PFU of virus (in 100 µl HBSS; pH 8) for 1 hour at 37°. The number of remaining PFU was determined by plaque assay on Vero cell monolayers without removing the inoculum (Chapter 3). Parallel control PRNTs with MVE-1-51 were carried out.

4.2.4 Selection of antigenic variants

Antigenic variants of MVE-1-51 were selected by Dr R. C. Weir or Dr J. T. Roehrig. Different methods of selection were used by each investigator.

Method 1 (R. C. Weir, personal communication)

Individual MVE-1-51 plaques were taken up in 5 µl HBSS (pH 8), amplified in Vero cells and plaque purified again. Virus (10^2-10^6 PFU) was incubated with neutralizing mAb, diluted 10-fold in HBSS (pH 8), at 37° for 1 hour, and used to infect Vero cell monolayers for plaque formation. Individual plaques were picked and amplified in Vero cells. A second round of selection was performed. Resistant plaques were amplified and used to infect Vero cell monolayers to produce virus stocks.

This precedure has been used previously to generate four E-1c variants (V1a, V3a, V4e and V5b), using as starting material a single stock of MVE-1-51 (S. H. Hartley and R. C. Weir, personal communication): V3a and V5b (used for virulence assay; see below) each contained an identical nucleotide substitution which gives a nonconservative change Ala 126→Glu; V1a and V4e contained the above substitution as well as additional changes at residues 125 and 355 respectively.

Method 2 (J. T. Roehrig, personal communication)

MVE-1-51 virus (10^4 PFU, grown in SW13 cells) was incubated with mAb (anti-E-1d, E-5b or E-7; 10-fold diluted in 5 ml EMEM) at 37° for 1 hour, and used to infect Vero cells in 25 cm^2 flasks. Culture medium was harvested at 6 days p.i.; aliquots (0.5 ml) were mixed with mAb (10-fold diluted, 5 ml) and used to infect Vero cells as before. Cytopathic effects in Vero cells were observed at 9-10 days p.i., at which time culture media were harvested. Aliquots of 0.5 ml were taken and passaged twice in Vero cell monoloyers in 6-well plastic trays without antibody. The virus stocks were harvested at 6 days p.i. and were used for plaque purification in the presence of mAb (10-fold diluted) to produce variants. These were tested for resistance to neutralization as follows: virus stocks (50 µl; diluted 10^{-1} in

EMEM) were incubated with selecting mAb (50 µl; diluted 10^{-2} in EMEM) at 37° for 2 hour and subjected to end-point dilution plaque assays on Vero cell monolayers. Anti-E-1d, E-5b and E-7 mAbs neutralized more than 99% of MVE-1-51; variants which were most resistant to anti-E-1d (60-100% resistant), anti-E-5b (21-60% resistant) and to anti-E-7 mAbs (57-94% resistant) were used for sequence analysis.

4.2.5 RNA extraction from MVE-infected Vero cells (Shine and Dalgarno, 1973)

RNA for sequence analysis was extracted from infected Vero cells (MOI≈0.1) in glass Petri dishes (diameter=15 cm) at 48 hours p.i. Vero cell monolayers were lysed in 4 ml each of ice-cold 6% sodium para-aminosalicylate (BDH Chemicals Ltd., Poole, England) and a mixture of redistilled phenol: m-cresol (BDH): 8-hydroxyquinoline (BDH) (80%: 10%: 0.1%; w/v/w), and extracted by vortexing thoroughly. Centrifugation was at 8000 rpm (Sorval SS34 rotor) for 10 min at 4°; the aqueous phase was extracted again with 0.8 ml of 15% NaCl and 2 ml of ice-cold phenol/cresol. Nucleic acids were precipitated with ethanol (2.5 volumes). Nucleic acid pellets were resuspended in NET buffer (0.12 M NaCl, 1 mM Na_2EDTA, 0.012 M Tris-HCl, pH 7.4) and mixed with an equal volume of 4 M LiCl. High mol. wt. RNA was pelleted after freeze-thawing and centrifugation for 10 min in an Eppendorf centrifuge. The RNA pellet was resuspended in NET buffer and precipitated with 4 M LiCl again. The resulting RNA was resuspended and precipitated twice with ethanol before dissolving in 5 mM Tris-HCl (pH 7.5); storage was at -70°. Nucleic acid content was quantitated by spectrophotometry; the yield per Petri dish was ≈0.2 mg of RNA.

4.2.6 Sequence analysis of M and E genes in MVE RNA

Dideoxy sequencing (Sanger *et al.*, 1977) was with RNA from MVE-infected Vero cells as template and HPLC-purified oligonucleotide primers (Bresatec, South Australia or Protein/DNA Facility, Australian National University) complementary to sequences in the MVE E and NS1 protein genes (Dalgarno *et al.*, 1986). Eight primers were used:

GTGTATGAATATTCC (2528-2542), AAGAGCTGCCAATCT (2222-2236), ATTGATCTGCTTGTC (2141-2155), CATGGTCCATCACTC (1980-1994), AACTCGACTGGTATG (1780-1794), TCACGATGCACTAGA (1600-1614), ACTTCATATTTGATG (1375-1389) and TCCCAGACAGTTAAA (974-988). Numbers indicate nucleotide positions in the genome of MVE-1-51 (Dalgarno *et al.*, 1986). Primer and template were annealed by incubating 2-5 µg of infected-cell RNA with 75 pmole of primer for 5 min at 56°. The mixture was quick-chilled and KCl was added to 80 mM. The final reaction mixture contained 50 mM Tris-HCl (pH 8.3); 8 mM $MgCl_2$; 50 mM KCl; 0.4 mM dithiothreitol; 50 µM dATP, dTTP and dGTP; 2.5 µM dCTP; 5 µCi α-^{32}P-dCTP; 0.2 µM ddCTP, 2.5 µM ddTTP, 5 µM ddATP or 2 µM ddGTP; 1.5 µl primer/template mixture; and 6 units of avian myeloblastosis virus (AMV) reverse transcriptase (Molecular Genetic Resources, Tempa, Florida, USA) in a final volume of 5 µl. Incubation was at 42.5° for 1 hour followed by addition of each dNTP to 5 µM and incubating a further 30 min at 42.5° to 'chase' polymerization of ^{32}P-labelled chains to completion. 20 µl of loading buffer containing 96% formamide (deionized), 25 mM Tris-borate (pH 8.3) and 0.5 mM Na_2EDTA was added to terminate the reaction. The mixture was incubated in a 90° waterbath for 2 min immediately prior to electrophoresis on 40 cm-long, 0.4 mm-thick, 6% polyacrylamide gels (acrylamide: bisacrylamide, 19:1). Gels were transferred to Whatman 3 MM paper, dried under vacuum at 80° and exposed to Fuji RX Medical X-ray film for autoradiography. MVE-1-51 RNA was sequenced in parallel as a control; ambiguous nucleotides were resolved in most cases by repeating sequencing reactions using alternate primers, different concentrations of dNTPs and primers, or higher annealing temperatures.

4.2.7 Virulence assays in mice (Monath *et al.*, 1980)

The basis of the virulence assay is to compare mortality ratios in 21-day old mice following ic and ip inoculation of ten-fold serial dilutions of virus. In this assay, virulent virus (e.g. MVE-1-51) gives similar mortality ratios after ip as after ic

inoculation (Lobigs *et al.*, 1988). However attenuated virus, despite being lethal after ic inoculatiion, shows a reduction in lethality after ip inoculation, implying a lack of neuroinvasiveness using a peripheral route of inoculation. The age of mice is crucial because mortality declines with age. The assay was performed on outbred Swiss mice (20-22 day old) randomized in groups of five. Virus to be tested was either undiluted or diluted 10-fold serially from 10^{-1} to 10^{-8} in PBS containing FCS (5%) and an inoculum of 30 µl used. Eighteen groups of mice were inoculated in total for each virus. Mice were examined daily for 14 days and deaths were recorded. LD50 values from ic and ip inoculations were calculated using the method of Reed and Muench (1934).

4.3 RESULTS

4.3.1 Sequence analysis of MVE variants selected by anti-E-1c

MVE-1-51 (prototype) virus stocks, derived from individually plaque purified clones, were incubated with anti-E-1c mAb (Table 3.1). Virus which escaped neutralization was isolated by plaque formation. Virus stocks grown from such plaques were incubated again with anti-E-1c mAb followed by plaque purification to produce variants. Variants P1c1, P4a1 and P5a1 were from separate plaque purified MVE-1-51 stocks (P1, P4, P5) and had thus arisen from independent mutations. The above steps were performed by Dr R. C. Weir. This procedure was also used with the anti-E-1d, E-5b, E-7 and E-8 mAbs but no antigenic variants were isolated (S. H. Hartley and R. C. Weir, personal communication).

To determine the resistance of variants P1c1, P4a1 and P5a1 to the selecting mAb, PRNT was performed. For MVE-1-51, 50% neutralization occurred at a 1/1000 dilution of anti-E-1c whereas less than 2% of P1c1, P4a1 and P5a1 was neutralized at the same dilution. Less than 30% of P1c1, P4a1 and P5a1 was neutralized at a 1/80 dilution of anti-E-1c. Thus there was more than a 10-fold difference between the 50% neutralization titres for MVE-1-51 and the E-1c variants.

For sequence analysis, P1c1, P4a1 and P5a1 were used to infect Vero cell monolayers. High mol. wt. RNA from infected cells was extracted for sequencing (Sanger *et al.*, 1977). Primers complementary to sequences in the NS1 and E genes and located 200-400 nucleotides apart were used. The nucleotide sequence of the entire M and E genes of the variants was obtained. RNA from MVE-1-51 infected cells was sequenced in parallel. 'Cross-bands' were encountered at particular nucleotides in the M and E genes. Sequencing with alternate primers and different concentrations of dNTPs or ddNTPs resolved some ambiguities. Approximately 5% of the sequence was cross-banded (Fig. 4.1) and could not be resolved. Since the cross-bands were at identical positions for the MVE variants and for MVE-1-51, it is likely that the variants were not different from the wild-type at these positions.

The sequence of MVE-1-51 E and M genes agreed with that reported by Dalgarno *et al.* (1986) except for a silent difference at position 2032 (G→A). E-1c variants P1c1, P4a1 and P5a1 each contained a different single nucleotide change in the E gene and no change in the M gene. These substitutions led to the following nonconservative amino acid substitutions: Phe 274→Val (1793, U→G) in P1c1, Ser 276→Arg (1801, C→A) in P4a1 and Ser 277→Ile (1803, G→U) in P5a1 (Table 4.1). The amino acids Phe 274, Ser 276 and Ser 277 in the E protein of MVE are therefore probably involved in determining resistance to anti-E-1c mAb.

4.3.2 Sequence analysis of E-1d, E-5b and E-7 variants

E-1d, E-5b and E-7 variants were provided by Dr J. T. Roehrig. The method used by him to generate variants involved incubating MVE-1-51 with the appropriate neutralizing mAbs and amplifying in Vero cells. Aliquots of the culture medium, harvested after 6 days, was incubated with mAb again and further amplified for 9-10 days. There were two additional passages in Vero cells without antibodies. Then the virus stocks were plaqued on Vero cells with the selecting mAb and the plaques amplified in Vero cells. Candidate variants were tested (by J. T. R.) for resistance to the

Figure 4.1 Nucleotide sequences of the M and E genes of MVE-1-51 and variant E-1c/P1c1. Sequences of the antigenic variants of MVE are compared with the prototype strain MVE-1-51 (top line; Dalgarno *et al.*, 1986). Those nucleotides that differ from the prototype sequence are indicated. Nucleotides which could not be unambiguously determined are designated N. Numbering is from the 5' nucleotide of the MVE-1-51 genome (Dalgarno *et al.*, 1986).

```
     → M       |         |         |         |         |         |         |         |         |
749  UCCAUCACAGUGCAGACUCAUGGUGAAAGCACUUUGGUCAACAAAAAGGAUGCCUGGCUGGAUUCCACGAAGGCCACGCGUUAUCUCACCAAAACAGAGA
MVE
P1c1

849  ACUGGAUUAUAAGAAAUCCUGGUUACGCGCUGGUGGCCGUUGUCCUUGGCUGGAUGCUGGGCAGCAACACUGGACAAAAAGUUAUUUUUACAGUGCUUUU
MVE                        N               N
P1c1                       N               N
                        → E
949  GCUCCUCGUUGCUCCUGCCUACAGUUUUAACUGUCUGGGAAUGAGCAGCCGUGAUUUCAUUGAAGGUGCUUCAGGAGCUACAUGGGUCGAUUUGGUGCUG
MVE                                                                                          N
P1c1                                                                                         N

1049 GAGGGCGACAGUUGCAUCACCAUCAUGGCCGCUGACAAACCCACCCUUGACAUAAGAAUGAUGAACAUUGAAGCCACCAAUCUUGCACUGGUUAGAAAUU
MVE       N
P1c1      N

1149 ACUGCUAUGCAGCUACUGUGUCAGACGUUUCUACGGUGUCAAACUGUCCUACUACAGGGGAGUCACACAACACGAAGCGGGCAGAUCACAAUUACUUGUG
MVE              N
P1c1             N

1249 CAAACGAGGUGUGACCGACAGAGGCUGGGGUAAUGGAUGUGGCUUGUUUGGUAAGGGGAGCAUUGACACAUGCGCAAAGUUCACCUGCUCUAACUCAGCU
MVE
P1c1

1349 GCGGGGAGACUUAUCUUACCUGAGGACAUCAAAUAUGAAGUUGGGGUUUUUGUUCACGGAUCAACGGACUCAACCAGUCAUGGAAAUUAUUCUACCCAAA
MVE
P1c1

1449 UUGGAGCUAACCAAGCAGUCAGGUUCACCAUUUCACCAAACGCUCCAGCCAUCACAGCAAAGAUGGGCGACUAUGGAGAAGUCACUGUGGAGUGUGAACC
MVE                                                                  N           N
P1c1                                                                 N           N

1549 GAGGAGUGGACUGAAUACAGAGGCCUACUACGUCAUGACCAUUGGAACGAAACACUUUCUAGUGCAUCGUGAGUGGUUCAAUGAUUUGCUCUUGCCAUGG
MVE                          N                                                   N
P1c1                         N                                                   N

1649 ACAUCACCUGCAAGCACGGAAUGGAGGAAUAGAGAAAUUCUCGUGGAGUUUGAAGAGCCACAUGCCACCAAACAAUCAGUGGUUGCCUUGGGUUCACAGG
MVE                                                    N N     N
P1c1                                                   N N     N

1749 AAGGAGCUUUGCACCAAGCUCUGGCUGGAGCCAUACCAGUCGAGUUUUCGAGCAGCACACUUAAACUCACUUCAGGACACCUUAAGUGUCGCGUGAAAAU
MVE                                                                  N
P1c1                                          G                      N

1849 GGAGAAAUUGAAACUGAAAGGAACCACUUAUGGGAUGUGCACAGAAAAAUUUACUUUCUCAAAGAAUCCAGCCGACACCGGUCAUGGCACGGUAGUUCUA
MVE
P1c1

1949 GAACUGCAGUACACCGGGAGUGAUGGACCAUGCAAAAUUCCAAUAUCCUCUGUAGCAAGUCUCAAUGACAUGACGCCUGUCGGGAGAAUGGUGACAGCUA
MVE                                                                                  A               N
P1c1                                                                                 A               N

2049 AUCCAUAUGUAGCUUCAUCAACUGCCAAUGCUAAAGUUCUGGUGGAGAUUGAACCACCCUUCGGAGACUCAUACAUUGUGGUAGGCAGGGGAGACAAGCA
MVE                                                    N
P1c1                                                   N

2149 GAUCAAUCACCACUGGCAUAAGGAGGGUAGUUCAAUUGGCAAAGCCUUCAGCACAACCUUGAAGGGAGCACAGAGAUUGGCAGCUCUUGGAGACACGGCG
MVE                                       N
P1c1                                      N

2249 UGGGACUUUGGAUCAGUAGGCGGAGUCUUCAAUUCAAUCGGAAAGGCAGUACACCAAGUCUUUGGAGGAGCAUUUAGAACCCUCUUUGGAGGAAUGUCAU
MVE
P1c1

2349 GGAUCAGCCCAGGUCUGCUGGGGGCGUUACUGCUAUGGAUGGGGGUCAAUGCUAGAGAUAAAUCAAUUGCUUUGGCUUUCCUAGCAACAGGAGGCGUUUU
MVE                              N                   N                                                   N
P1c1                             N                   N                                                   N

2449 GUUGUUCCUGGCCACAAAUGUCCAUGCU
MVE
P1c1
```

[a] E-1c variants were selected by Dr R. C. Weir, E-1d, E-5b and E-7 variants were selected by Dr J. T. Roehrig (Materials and Methods).

[b] Variants were sequenced through the entire E and M genes and substitutions were determined by comparison with the MVE-1-51 sequence. Numbering is from the 5' nucleotide of the MVE-1-51 genome (Dalgarno *et al.*, 1986).

[c] Substitutions which occur in variants selected by different mAbs are bracketed. Numbering is from the N-terminal amino acid of the MVE E protein (Dalgarno *et al.*, 1986).

[d] Conservative amino acid substitution; the following changes are designated as conservative: Arg = Lys, Ser = Thr, Asp = Glu, Gln = Asn, Val = Leu = Ile = Met, Ala = Gly, Ala = Val, Tyr = Phe.

TABLE 4.1

Sequence analysis of MVE antigenic variants

Epitope	Variant[a]	Nucleotide substitutions[b]	Amino acid substitutions[c]
E-1c	P1c1	1793 (UUU→GUU)	Phe 274→Val
	P4a1	1801 (AGC→AGA)	Ser 276→Arg
	P5a1	1803 (AGC→AUC)	Ser 277→Ile
E-1d	VAR1	1373 (GAC→AAC)	[Asp 134→Asn]
		1760 (CAC→UAC)	His 263→Tyr
		1903 (ACU→ACG)	-
		2045 (GCU→ACU)	[Ala 358→Thr]
	VAR3	1373 (GAC→AAC)	[Asp134→Asn]
		1760 (CAC→UAC)	His 263→Tyr
		2045 (GCU→ACU)	[Ala 358→Thr]
	VAR5	1373 (GAC→AAC)	[Asp 134→Asn]
		1760 (CAC→UAC)	His 263→Tyr
		1903 (ACU→ACG)	-
		2045 (GCU→ACU)	[Ala 358→Thr]
E-5b	VAR1	1373 (GAC→AAC)	[Asp 134→Asn]
		1562 (AAU→GAU)	Asn 197→Asp
		2045 (GCU→ACU)	[Ala 358→Thr]
	VAR3	1373 (GAC→AAC)	[Asp 134→Asn]
		1562 (AAU→GAU)	Asn 197→Asp
		2045 (GCU→ACU)	[Ala 358→Thr]
	VAR5	1124 (ACC→GCC)	Thr 51→Ala
		1373 (GAC→AAC)	[Asp 134→Asn]
		2045 (GCU→ACU)	[Ala 358→Thr]
E-7	VAR1	1373 (GAC→AAC)	[Asp 134→Asn]
		1907 (ACU→UCU)	Thr 310→Ser[d]
		2045 (GCU→ACU)	[Ala 358→Thr]
	VAR2	1373 (GAC→AAC)	[Asp 134→Asn]
		2045 (GCU→ACU)	[Ala 358→Thr]
	VAR4	1373 (GAC→AAC)	[Asp 134→Asn]
		2045 (GCU→ACU)	[Ala 358→Thr]

selecting mAbs. E-1d, E-5b and E-7 variants were selected. No E-1c or E-8 variants were obtained using this method (J. T. R., personal communication). The three variants out of each group which showed most resistance to the selecting mAbs were chosen for sequence analysis (see below).

Sequence analysis of E-1d variants

The three E-1d variants chosen for sequence analysis (VAR1, VAR3, VAR5) were 85, 100 and 60% resistant to anti-E-1d. Sequence analysis was as described above. The seed stock of MVE-1-51, used by J. T. R. to generate the antigenic variants, was sequenced in parallel. This stock had the same silent difference from the published sequence at position 2032 (G→A) (Dalgarno *et al.*, 1986). The E-1d variants each contained three or four nucleotide substitutions, leading to three nonconservative amino acid substitutions (Table 4.1). The same substitions, Asp 134→Asn, His 263→Tyr and Ala 358→Thr, were found in all three variants and were derived from identical nucleotide changes at 1373 (G→A), 1760 (C→U) and 2045 (G→A). VAR1 and VAR5 contained, in addition, a silent nucleotide substitution at position 1903 (U→G).

Sequence analysis of E-5b variants

E-5b variants used for sequence analysis (VAR1, VAR3, VAR5) were 51%, 21% and 60% resistant to anti-E-5b. VAR1, VAR3 and VAR5 each contained three changes leading to nonconservative amino acid substitutions (Table 4.1). The substitutions Asp 134→Asn and Ala 358→Thr were common to all three variants and derived from identical nucleotide changes at 1373 (G→A) and 2045 (G→A). VAR1 and VAR3 also contained an additional change Asn 197→Asp (A→G) while VAR5 contained the substitution Thr 51→Ala (A→G).

Sequence analysis of E-7 variants

E-7 variants used for sequence analysis (VAR1, VAR2, VAR4) were 62, 57 and 94% resistant to anti-E-7. VAR1, VAR2 and VAR4 each contained two or three nucleotide changes (Table 4.1). The nonconservative substitutions Asp 134→Asn and

Ala 358→Thr were found in all three variants and were derived from identical nucleotide changes at 1373 (G→A) and 2045 (G→A). VAR1 also contained the conservative substitution Thr 310→Ser (A→U). VAR2 and VAR4 had no change at residue 310 or at any additional residues, suggesting Thr 310 was probably not a determinant of resistance to anti-E-7 mAb.

Neutralization tests using anti-E-1d, E-5b and E-7 mAbs

Each of the E-1d, E-5b and E-7 variants contained two identical changes in E (Asp 134→Asn and Ala 358→Thr) as a result of the same nucleotide changes at 1373 (G→A) and 2045 (G→A). Neither of these changes occurred in the E-1c variants which were selected using a different procedure (see Discussion). The question of whether the changes at Asp 134 and Ala 358 were responsible for the resistance of E-1d and E-5 variants may have been resolved by performing PRNT on E-7 VAR2 or VAR4, which contained only changes at Asp 134 and Ala 358, using anti-E-1d and E-5b mAbs. There is also a natural isolate of MVE (TC 123130) which is identical to MVE-1-51 in E sequence except for a single Asp 134→Asn change (Lobigs *et al.*, 1988). This strain could have been used in examining the importance of Asp 134 to epitopes E-1d, E-5b and E-7. However efforts to study this in cross-neutralization tests with mAbs specific for epitopes E-1d, E-5b and E-7 were unsuccessful due to the low titres of some of the mAbs sent from the USA (see Materials and Methods). As a result of the loss in neutralization activity of mAbs in transit, the importance of Asp 134 and Ala 358 to epitopes E-1d, E-5b and E-7 could not be determined.

4.3.3 Virulence assays in mice (Monath *et al.*, 1980)

The virulence of E-1c variants P1c1, P4a1, P5a1 and V5b (the latter provided by R. C. W.; see Materials and Methods), and of E-1d/VAR3, E-5b/VAR5 and E-7/VAR4 was examined (see Table 4.2). Virus stocks were diluted in serial 10-fold steps (from 10^{-1} to 10^{-8}), and aliquots of 30 µl inoculated into five mice by ic or ip routes. MVE-1-51 was assayed as the control. Deaths were recorded over 14 days for each

TABLE 4.2

Virulence of MVE variants in mice

Virus	Amino acid difference in E[a]	Titre[b]	ic LD50[c]	ip LD50[c]	ic/ip LD50 ratio	Virulence[d]
MVE-1-51	-	$1x10^6$	$10^{-7.2}$	$10^{-6.5}$	0.2	virulent
E-1c/P1c1	Phe 274→Val	$7x10^5$	$10^{-6.1}$	$10^{-5.9}$	0.6	virulent
E-1c/P4a1	Ser 276→Arg	$1x10^6$	$10^{-6.7}$	$10^{-4.9}$	0.02	intermediate?
E-1c/P5a1	Ser 277→Ile	$7x10^5$	$10^{-6.2}$	$10^{-3.0}$	0.0006	intermediate
E-1c/V5b	Ala 126→Thr	$5x10^6$	$10^{-8.2}$	$10^{-7.9}$	0.5	virulent
E-1d/VAR3	Asp 134→Asn His 263→Tyr Ala 358→Thr	$3x10^6$	$10^{-7.3}$	$10^{-6.3}$	0.1	virulent
E-5b/VAR5	Thr 51→Ala Asp 134→Asn Ala 358→Thr	$3x10^6$	$10^{-8.3}$	$10^{-7.4}$	0.1	virulent
E-7/VAR4	Asp 134→Asn Ala 358→Thr	$1x10^6$	$10^{-8.0}$	$10^{-7.8}$	0.6	virulent

[a] From wild-type sequence; see Table 4.1.

[b] Titre of each virus was determined by plaque assay on Vero cell monolayers.

[c] LD50 values for ip or ic inoculation were calculated for each virus by the method of Reed and Muench (1934).

[d] Virulence of each virus was assessed on the basis of ic/ipLD50 ratios (Monath *et al.*, 1980).

group of mice (Fig. 4.2). Virulence was assessed by comparison of ic and ip LD50 values for each virus (Monath *et al.*, 1980; Table 4.2).

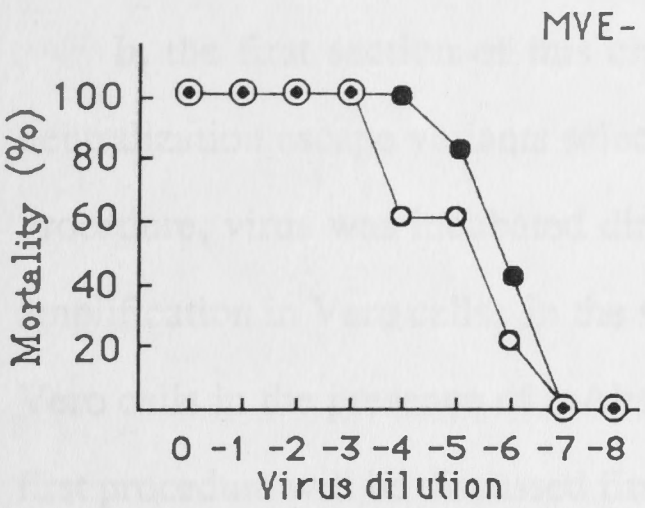
MVE-1-51 and variants E-1c/P1c1, E-1c/V5b, E-1d/VAR3, E-5b/VAR5 and E-7/VAR4 differed no more than 10-fold in ic and ip LD50 values and were therefore considered virulent. By contrast, E-1c variants P5a1 and P4a1 showed ic/ip LD50 ratios of 0.0006 and 0.02 respectively indicating attenuation. P5a1, changed from Ser 277 to Ile, is of 'intermediate' virulence according to the criteria of Monath *et al.* (1980). P4a1, changed at an adjacent position (Ser 276→Arg), was also associated with some attenuation: the ic/ip LD50 ratio was approximately 10-fold greater than MVE-1-51. This attenuation is also evident from Fig. 4.2 which shows a 'prozone' effect on ip inoculation at low dilutions of virus (10^0, 10^{-1}, 10^{-2}; 3 survivors out of 14 mice). Monath *et al.* (1980) have also noted that SLE isolates of intermediate virulence show a 'prozone' effect.

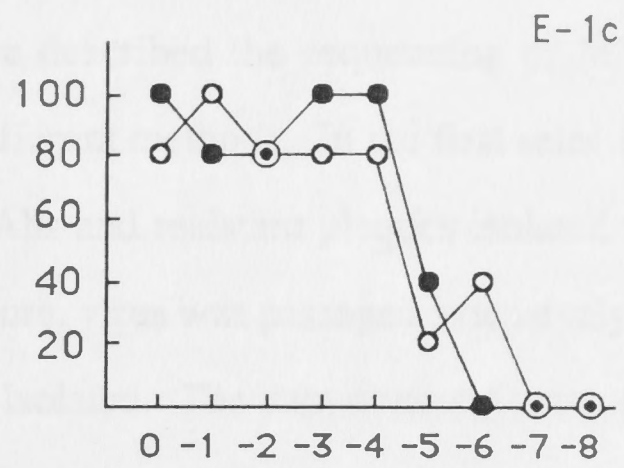
4.3.4 Summary

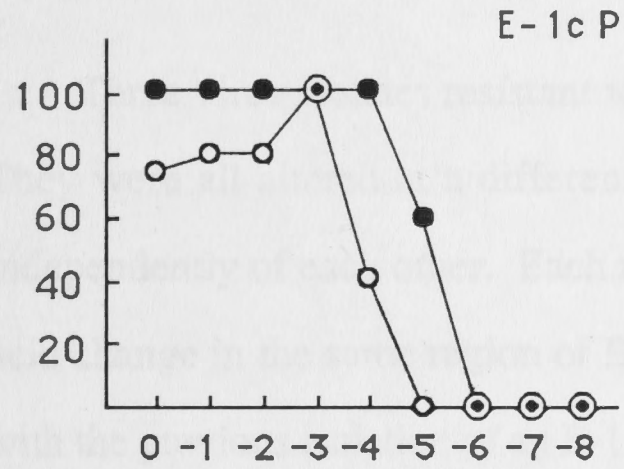
Changes in E gene sequence of neutralization escape variants of MVE-1-51 were used to map neutralization epitopes on the MVE E protein. Variants selected with the anti-E-1c mAb had single nonconservative amino acid changes at Phe 274, Ser 276 and Ser 277. Variants resistant to anti-E-1d, E-5b and E-7 mAbs were selected using a different method from those resistant to anti-E-1c. These contained several changes in E including the common changes Asp 134→Asn and Ala 358→Thr as well as His 263→Tyr for E-1d variants and Thr 51→Ala or Asn 197→Asp for E-5b variants. One E-7 variant contained a Thr 310→Ser change; the other E-7 variants did not.

Seven variants were tested for virulence in 21-day old mice. The ic/ipLD50 ratios indicated that two of the seven showed reduced virulence. The ic/ipLD50 ratios for E-1c/P4a1 and E-1c/P5a1 were approximately 10 and 1000-fold less than that for MVE-1-51. These variants had single nonconservative changes in E at residues 276 and 277 respectively.

Figure 4.2 Virulence of MVE-1-51 and antigenic variants in mice. MVE-1-51 and antigenic variants were tested for virulence in three week-old mice (Monath *et al.*, 1980). Virus (30 µl) diluted from 10^0 to 10^{-8} was used to inoculate mice in groups of five by the ip or ic route. Deaths were recorded over 14 days. Mortality ratio (%) at each dilution of virus is plotted. Inoculation by the ic and ip routes is indicated by dots and open circles respectively. Only four mice were inoculated ip with undiluted E-1c/P4a1; hence the mortality of 75%. One mouse in the group of five inoculated ic with E-7/VAR4 at 10^{-6} dilution died within two days and was disregarded; hence the mortality of 50%.


MVE-1-51
Mortality (%)
100
80
60
40
20
0 -1 -2 -3 -4 -5 -6 -7 -8
Virus dilution


E-1c P1c1
100
80
60
40
20
0 -1 -2 -3 -4 -5 -6 -7 -8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80
60
40
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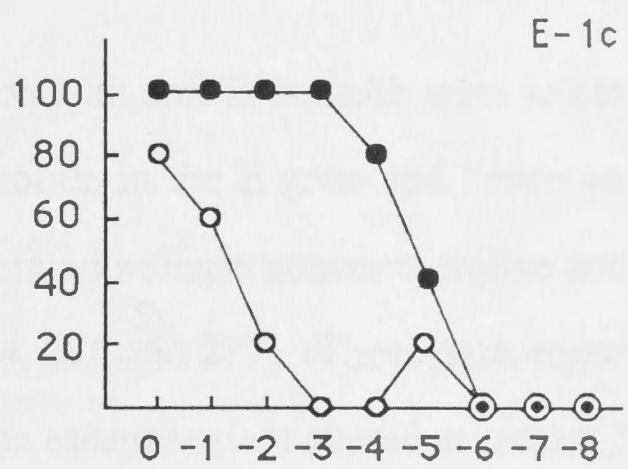
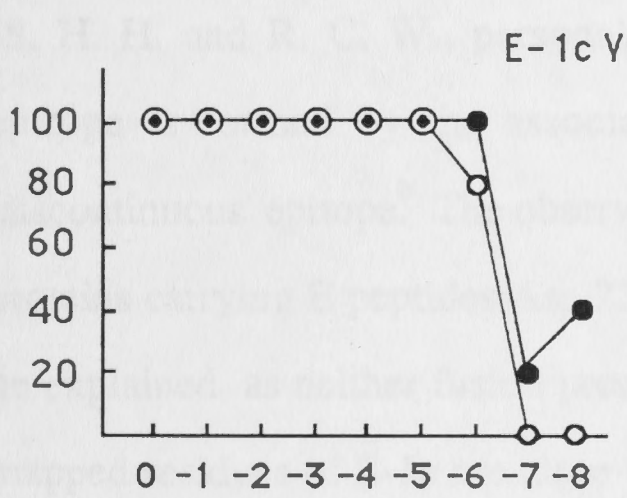
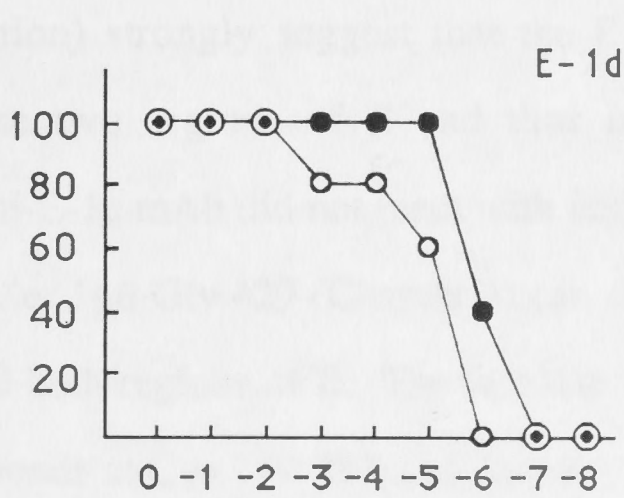
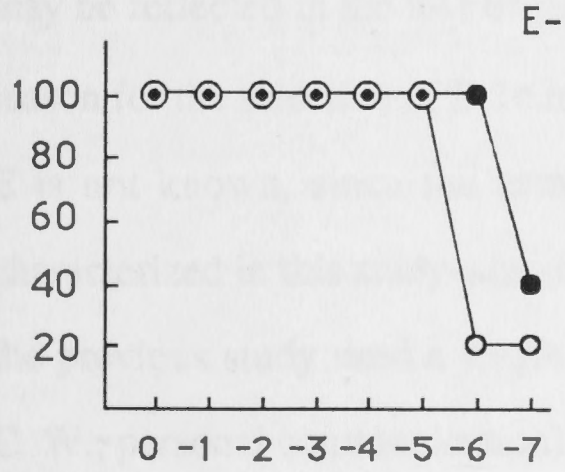
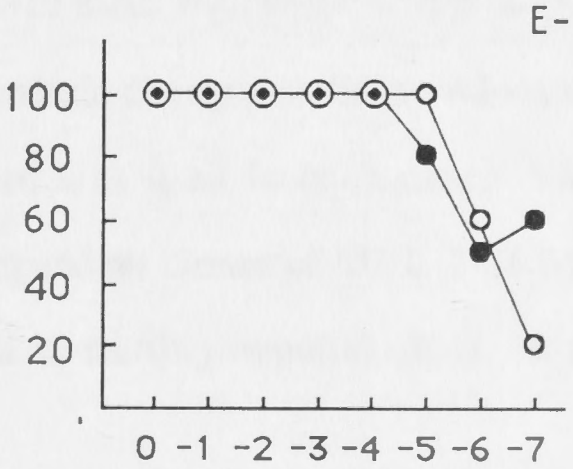
0 -1 -2 -3 -4 -5 -6 -7 -8


E-1c P5a1
100
80
60
40
20
0 -1 -2 -3 -4 -5 -6 -7 -8


E-1c Y5b
100
80
60
40
20
0 -1 -2 -3 -4 -5 -6 -7 -8


E-1d VAR3
100
80
60
40
20
0 -1 -2 -3 -4 -5 -6 -7 -8


E-5b VAR5
100
80
60
40
20
0 -1 -2 -3 -4 -5 -6 -7


E-7 VAR4
100
80
60
40
20
0 -1 -2 -3 -4 -5 -6 -7

4.4 DISCUSSION

Neutralization epitopes in the MVE E protein

In the first section of this chapter we have described the sequencing of MVE neutralization escape variants selected by two different methods. In the first selection procedure, virus was incubated directly with mAbs and resistant plaques isolated for amplification in Vero cells. In the second procedure, virus was passaged extensively in Vero cells in the presence of mAbs and variants isolated. The data obtained using the first procedure will be discussed first.

Three virus isolates resistant to neutralization with anti-E-1c mAb were selected. They were all altered at a different single nucleotide in the E gene and hence arose independently of each other. Each mutation generated a single nonconservative amino acid change in the same region of E (residues 274, 276 and 277). These data, together with the previous isolation of an E-1c neutralization escape variant altered at residue 126 (S. H. H. and R. C. W., personal communication) strongly suggest that the E-1c epitope is formed by the association of these two regions of E and thus is a 'discontinuous' epitope.* The observation that anti-E-1c mAb did not react with fusion proteins carrying E peptides Asp 22-Val 272 or Ser 146-Gly 429 (Chapter 3) can thus be explained, as neither fusion protein contained both regions of E. The fact that the mapped residues of E-1c are close to disulfide bonds at Cys 190-288 and Cys 60-121 may be reflected in the loss of reactivity of anti-E-1c mAb with reduced E protein. The reason for the selection of E-1c mutants with amino acid changes at different locations in E is not known, since the same basic procedure was used in each case. Variants characterized in this study were derived from independent clones of MVE-1-51 whereas the previous study used a single MVE-1-51 stock as starting material (S. H. H. and R. C. W., personal communication).

Anti-E-1c mAb was raised against MVE-1-51; it is type-specific showing no cross-reactivity with other members of the JE serogroup (Table 3.1). Thus anti-E-1c would

* However, we cannot discount the possibility that changes in one region of the E protein can influence the folding and hence the mAb binding in a quite distinct region.

be expected to recognize an epitope which differed in sequence between MVE and the closely related virus JE. The regions of E between residues 270 and 280, and around residue 126 show strong variations between flaviviruses in general and between members of the JE serogroup (Fig. 4.3). Both regions differ markedly between MVE and JE which are 82% identical overall in the amino acid sequence of E. The sequences of the E-1c epitope therefore show the expected divergence between MVE and other flaviviruses. It is of interest that two other closely related members of the JE serogroup, WN and KUN, are identical in sequence in the regions defined by anti-E-1c.

Fig. 4.4 is a hydropathy profile of the MVE E protein. In the primary amino acid sequence, residues 274, 276 and 277 are adjacent to a strongly hydrophilic region. A similar hydropathy profile is seen for YF in the same region (Dalgarno *et al.*, 1986). The amino acids in this hydrophilic stretch are strongly conserved as to sequence between members of the JE serogroup (Fig. 4.3) suggesting an important function. Residue 126 is within a variable region, as noted above. It is adjacent to the possible fusion sequence (Chapter 1; see below). Considered together, the data suggest that the epitope defined by anti-E-1c may be important not only as a binding site for neutralizing antibody but also in virus entry. Data showing alterations in viral virulence for E-1c variants support this view (see below).

Sequence analysis of E-1d, E-5b and E-7 variants revealed more than one amino acid substitution in each. Two common changes, both nonconservative, occurred in all variants; these were Asp 134→Asn and Ala 358→Thr. The explanation for these unexpected results may lie in the method used for selection of these variants which involved a series of passages of virus in Vero cells before plaque purification. It seems possible that the changes observed at residues 134 and 358 were 'cell-selected' during this process rather than mAb-selected. Lobigs *et al.* (1990) and A. Nestorowicz (unpublished results) have previously noted that specific changes in the MVE E protein are selected during passaging of MVE in cell culture. Considering the change at residue

Figure 4.3 Co-listing of E protein sequences of ten flaviviruses (adapted from Fig. 1.3). References for the E protein sequences of flaviviruses are cited in Fig. 1.3. The Asn-X-Thr/Ser tripeptides are underlined and the C-terminal hydrophobic regions are overlined. Strictly conserved cysteines are indicated by asterisks. Amino acid substitutions which occurred in the neutralization escape variants of MVE are shown together with the epitopes defined by the corresponding selecting mAbs.

```
                                                                E-5b
          *         |         |         *         |         |A        *         |    *    |         90
MVE       FNCLGMSSRDFIEGASGATWVDLVLEGDSCITIMAADKPTLDIRMMNIEATNLALVRNYCYAATVSDVSTVSNCPTTGESHKTKRADHNY
JE        ......GN.......................L....N......V..I....SQ..E..S...H.S.T.I...AR......A.VE....SS.
KUN       .......N...L..V................V...SK....I.VK...M..A...E..S...L.T..EL..KAA...M..A.ND....PSF
WN        .......N...L..V................V...SK....I.VK...M..A...D..S...L.S...L..RAA...M..A.NE....PAF
SLE       .....T.N...V........I......G..V.V..PE.....FKV.KM...E..T..K...E..LDTL...AR......A.N...S.PTF
YF-17D    AH.I.ITD......VH.G...SAT..Q.K.V.V..P...S...SLETVAIDRP.E..KV..N.VLTH.KINDK..S...A.LAEENEGDN
DEN-1     MR.V.IGN...V..L.......V...HG..V.T..K.......ELLKT.V..P.VL.KL.IE.KI.NTT.D.R...Q..ATLVEEQ.T.F
DEN-2     MR.I.I.N...V..V..GS...I...HG..V.T..KN.....FELIKT..KQP.TL.K..IE.KLTNTT.E.R...Q..PSLNEEQ.KRF
DEN-4     MR.V.VGN...V..V..GA.......HGG.V.T..QG.....FELTKTT.KEV..L.T..IE.SI.NIT.ATR...Q..PYLKEEQ.QQ.
TBE       SR.THLEN...VT.TQ.T.R.T....LGG.V..T.EG..SM.VWLDA.YQE.P.KT.E..LH.KL..TKVAAR...M.PATLAEEHQGGT

                                        E-1c  E-1d,E-5b,E-7
          *         |    *    |    *    |*    E    |   N     |         |         |         |        180
MVE       LCKRGLTDRGWGNGCGLFGKGSIDTCAKFTCSNSAAGRLILPEDIKYEVGVFVHGSTDSTSHGNYSTQIGANQAVRFTISPNAPAITAKM
JE        V..Q.F.........................S.TSK.I..T.Q..N......I....T.T.EN.....A.V..S..AK...T....S..L.L
KUN       V..Q.VV........................A..TK.T..T..K.N.....AI....P.TVE.....F..T..A..G..S.T.A..SY.L.L
WN        V..Q.VV........................A.TTK.T.WI.QK.N.....AI....P.TVE...K-------T..G..S.T.S..SY.L.L
SLE       V...DVV........................K.K.T.KT..R.N.....AI.............E...K...A......Q..SF..N.
YF-17D    A...TYS..............VA......AK.MSLFEVDQTK.Q.VIRAQL.VGAKQENWNTDIK--------TLKFDALSGSQEVEF
DEN-1     V.R.TFV..............LI.....K.VTKLE.KIVQY.NL..S.I.T..TGDQHQVGNETTEHG-----TTA..T.Q..TSEIQL
DEN-2     ...HSMV..............G.V...M...KKNME.KVV...NLE.TIVITP.SGEEHAVGNDTGKHG-----KEIK.T.QSSITE.EL
DEN-4     I.R.DVV..............GVV.....S..GKIT.N.VRI.NLE.T.V.T..NGDTHAVGNDT.NHG-----.TAM.T.RS.SVEV.L
TBE       V...DQS......H.........VA.V.AA.EAKKKATGHVYDAN.IVYT.K.EPH.GDYVAA.ETHSGRKTA--S....SEKTIL.--.

                        E-5b
                  *      D  |                 |         |         |         |         |        261
MVE       GDYGEVTVECEPRSGLNTEAYY------VMTIGTKHFLVHREWFNDLLLPWTSPASTE---WRNREILVEFEEPHATKQSVVALGSQEGA
JE        .......LD...........F.-------...V.S.S........H..A......S..A---.....L.M....A..............G
KUN       .E......D......ID.S...-------...V............M..N...S.AE.NV---.....T.M............I........
WN        .E......D......ID.S...-------..SV.E.S........M..N...S.AG..T---.....T.M......................
SLE       .E..T..ID..A...I...D..-------.F.VKE.SW..N.D..H..N.......T.D---.....T............T.........R
YF-17D    IG..KA.L..QVQTAVDFGNS.------IAEME.ESWI.D.Q.AQ..T...Q.GSGGV---..EMHH.....P...ATIR.L...N...S
DEN-1     T...AL.LD.S..T..DFNEMV------LL.MEK.SW...KQ..L..P.....G...SQET.NRQDL..T.KTA..K..E..V.......
DEN-2     TG..T..M..S..T..DFNEMV------LLQMED.AW....Q..L..P...LPG.D.QGSN.IQK.T..T.KN...K..D..V.......
DEN-4     P....L.LD...GLE.YFNEMI------L.KMKK.TW...KQ..LN.P....AG.D.S.VH.NYK.RM.T.KV...KR.D.TV.E.....
TBE       .E..D.SLL.RVA..VDLAQTVILELDKTVEHLPTAWQ...D.....A...KHEFAQN---.N.A.R....GA...V.MD.YN..D.T.V

          E-1d            E-1c                                  E-7
           Y      |       V RI  |         * |         |    *    S          |         |     *   |    347
MVE       LHQALAGAIP---VEFSSSTLKLTSGHLKCRVKMEKLKLKGTTYGMCTEKFTFSKN-PADTGHGTVVLELQYTGSDGPCKIPISSVASLN
JE        .........V---..Y..-SV..........L..D..A.............S.A..-..........I..S.S..........V.......
KUN       ..........---.....N.V.................Q.......V.SKA.R.LGT-...................T...............
WN        ..........---.....N.V.................Q.......V.SKA.K.ART-...................T.....V.........
SLE       PAT.......---ATV.....T.Q.......A.LD.V.I........DSA.....-.T.......IV.......N...RV...VT.N.M
YF-17D    .KT..T..MRVTKDTNDNNLY..HG..VS....LSA.T....S.KI..D.MF.V.-.T........MQVKVSKGA-..R..VIVADD.T
DEN-1     M.T..T..TE----IQT.G.TTIFA......L..D..T...MS.V...GS.KLE.E-V.E.Q....LVQVK.E.T.A.....F..QD-EK
DEN-2     M.T..T..TE----IQM..GNL.FT......LR.D..Q...MS.S...G..KIV.E-I.E.Q...I.IRV..E.DGS.....FEIMD-.E
DEN-4     M.S.....TE----VD.GDGNHMFA.....K.R....RI..MS.T..SG..SID.E-M.E.Q...T.VKVK.E.AGA...V..EIRD-V.
TBE       .LK....VP.---AHIEGTKYH.K...VT.E.GL....M..L..T..DKTKFTW.RA.T.S..D...M.VTFS.TK-..R..VRA..HSP

             E-1d, E-5b, E-7
           |       T |         |         |         |         |         |         |         |    437
MVE       DMTPVGRMVTANPYVASSTANAKVLVEIEPPFGDSYIVVGRGDKQINHHWHKEGSSIGKAFSTTLKGAQRLAALGDTAWDFGSVGGVFNS
JE        .......L..V..F..T.S..S.....M.........................A..TL.............................I......
KUN       .L.....L..V..F.SV........I.L..............EQ........S.........TA.............................T.
WN        .L.....L..V..F.SVA...S...I.L..............EQ........S.........T...R.....................T.
SLE       .L.....L..V..FISTGG..N..MI.V..............TT...Y.............LA..W.......V..........I......
YF-17D    AAINK.IL..V..IASTNDDE--..I.VN........I.....SRLTYQ..........L.TQ.M..VE...VM.......S.A..F.T.
DEN-1     GV.QN..LI....I.TDKEKP--.NI.A.....E......A.E.ALKLS.F.K......M.EA.AR..R.M.I..........I....T.
DEN-2     KRHVL..LI.V..I.TEKDSP--.NI.A.........II.VEPG.LKLN.F.K.....QM.E..MR..K.M.I..........L....T.
DEN-4     KEKV...IISST.LAENTNSV--TNI.L.R.-L.....I.V.NSALTL..FRN......M.E..YR..K.M.I..E..........L.T.
TBE       .VNVAM-LI.P..TIENNGGG--.FI.MQL.P..NI.Y..----ELS.Q.FQK.....RV.QK.K..IE..T.I.EH......A..FLS.


          |         |         |         |         |         |         |         |         |         501
MVE       IGKAVHQVFGGAFRTLFGGMSWISPGLLGALLLWMGVNARDKSIALAFLATGGVLLFLATNVHA
JE        .........................TQ..M.............R..............V.........
KUN       V...I.........S........TQ............I....R....T...V.......SV....
WN        V...I.........S........TQ............I....R...MT...V.......SV....
SLE       .........................TQ............LQ...R..S.TL..V..I.I....S.Q.
YF-17D    V..GI.T...S..QG....LN..TKVIM..V.I.V.I.T.NMTMSMSMILV.VIMM..SLG.G.
DEN-1     V..LI..I..T.YGV..S.V..TMKIGI.I..T.L.L.S.ST.LSMTCI.V.M.T.Y.GVM.Q.
DEN-2     ....L.....AIYGAA.S.V..TMKI.I.VIIT.I.M.S.ST.LSVSLVLV.V.T.Y.GAM.Q.
DEN-4     L.........SVYT.M...V..MIRI.I.F.V..I.T.S.NT.M.MTCI.V..IT...GFT.Q.
TBE       ......T.L....NSI...VGFLPKL...VA.A.L.L.M.NPTMSMS..LA..LV.AMTLG.G.
```

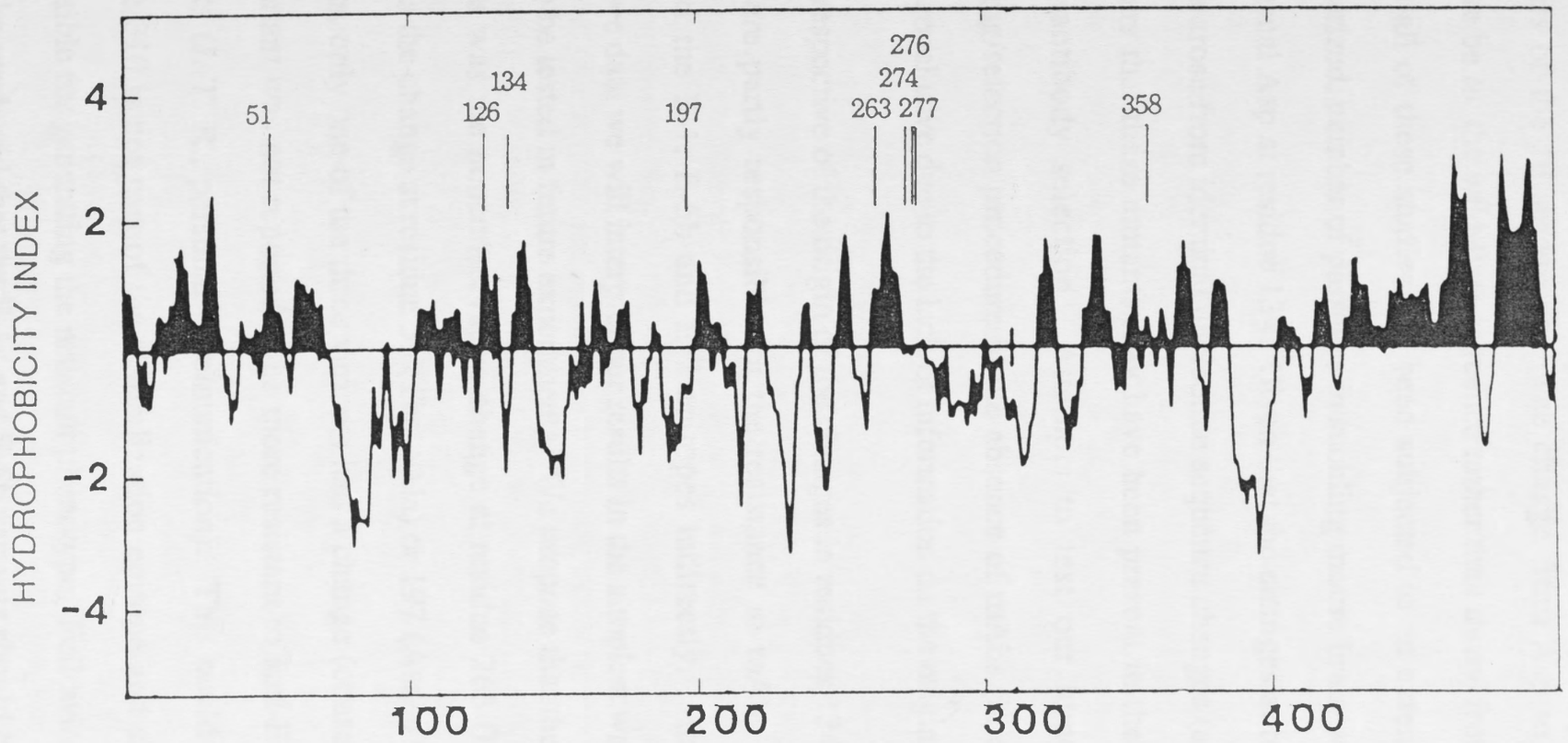


Figure 4.4 Hydropathy profile of the MVE E protein (adapted from Dalgarno *et al.*, 1986). The method of Kyte and Doolitle (1982) was used to determine hydropathy profile of the MVE E protein. Positions where amino acid substitutions occurred in the neutralization escape variants of MVE are indicated.

134, it is noteworthy that Asn 134 is encoded in the E gene of all eleven MVE isolates which have been examined except MVE-1-51 (Lobigs *et al.*, 1988) as well as in all other members of the JE serogroup. The change from Asp to Asn at this position may therefore be *to* the wild-type sequence rather than *away* from it. The MVE-1-51 stock used in all of these studies had been subjected to an extensive, though incompletely characterized, number of passages in suckling mouse brain which may have selected for the unusual Asp at residue 134. Given that the changes at residue 134 in the antigenic variants arose from identical nucleotide sequence changes (as did those at residue 358), it appears that these mutants may have been present in the parental MVE-1-51 stock before antibody selection. Attempts to test our hypothesis by repeating the passaging/selection procedure in the absence of mAbs (in our laboratory) have so far been inconclusive due to the lack of information on the original experimental conditions.

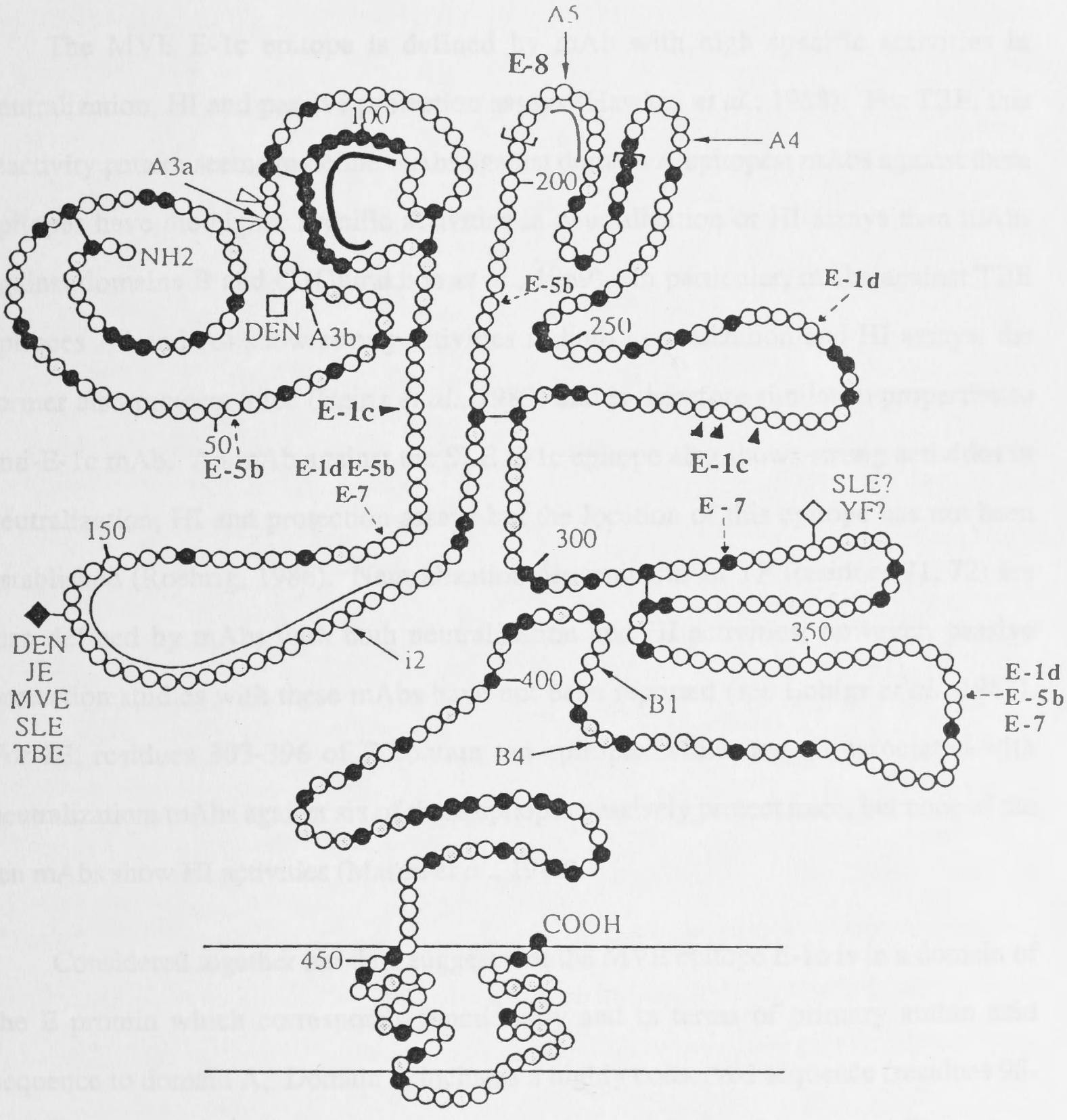
Irrespective of the origin of the changes in residues 134 and 358, it is possible that they were partly responsible for the resistance to mAbs and/or that these changes affected the E-1, E-5b and E-7 epitopes indirectly. However, in the absence of definitive data we will interpret our results in the simplest way; this interpretation would need to be tested in future experiments. We propose that the crucial change in the E-1d variants was the nonconservative change at residue 263 (His→Tyr), and in the E-5b variants the change at residue 51 (Thr→Ala) or 197 (Asn→Asp). In the case of the E-7 variants, only one of the three variants had a change (conservative) at residue 310 and this mutant was not reported to be more resistant to anti-E-7 mAb than the other two variants (J. T. R., personal communication). This could be interpreted as follows: residue 310 is not part of the neutralization epitope and changes at 134 and 358 are responsible for generating the resistant phenotype. Following from this interpretation, it would be predicted that the E-1d and E-5b variants should be resistant to neutralization with anti-E-7 mAb as well as to anti-E-1d and E-5b mAbs respectively. Although identification of the determinants of these three epitopes has not been achieved, the variants focus attention on a limited number of residues that may affect neutralization.

The MVE E protein in relation to the TBE model

From this study and the work described in Chapter 3, we have mapped determinants of the E-1c epitope to residues 126, 274, 276 and 277 in the MVE E protein and the E-8 epitope to between residues 201 and 224. Since the TBE E protein is the most completely mapped flavivirus E protein in terms of neutralization epitopes, it is appropriate to examine our data in relation to those of Mandl *et al.* (1989b). As previously noted, the TBE E protein may be subdivided into three domains based on data from CBAs, protein fragmentation studies and epitope mapping (Fig. 4.5). Domain A contains neutralization, protective and HI epitopes with flavivirus-group, complex and subtype specificities; it is sensitive to conformation changes (induced by SDS or reduction of disulfides) and is altered in conformation at pH 6 as judged by protease cleavage patterns and by the reactivity of domain A-specific mAbs with E (Guirakhoo *et al.*, 1989). Domain B also contains neutralization, protective and HI epitopes with both complex and type specificities. It can be isolated as a protease-resistant 'core' of approximately 9K, and loses antigenic reactivity after irreversible reduction; reactivities of mAbs against domain B are not affected at pH 6. Domain C contains a neutralization epitope and HI epitopes with complex, type and subtype specificities. It is resistant to irreversible reduction and incubation at pH 6 but loses reactivity on tryptic digestion.

The MVE E-1c and E-8 epitopes fall into domain A, which comprises 'left-hand' (residues 50-130) and 'right-hand' (the loop between disulfide bond at Cys 190-288) components (Fig. 4.5). E-8 is in the right-hand loop; residues defining E-1c are in both left and right components, giving support to the 'discontinuous' nature of domain A. Epitopes E-1c, E-1d, E-5b and E-8 have been characterized by CBAs as a continuum of overlapping epitopes; they are all sensitive to incubation at pH 6 (J. T. R., personal communication). Thus it is not surprising that E-1c, E-8 and the changes in E-1d and E-5b variants all fall in domain A, with the exception of Thr 358 which is in domain B (Fig. 4.5). All these MVE epitopes, with the exception of E-8, map to positions in

Figure 4.5 The TBE E model showing positions of neutralization determinants for TBE, YF and MVE (adapted from Fig. 1.4). Solid circles indicate amino acids completely conserved in flaviviruses and open circles indicate nonconserved amino acids. The neutralization determinants of TBE are indicated by arrows and those of YF by open arrowheads. Amino acids in the TBE E protein corresponding to the neutralization determinant E-1c of MVE are indicated by solid arrowheads (see Fig. 4.4). Positions corresponding to substitutions in MVE neutralization escape variants selected by anti-E-1d, E-5b and E-7 mAbs are indicated by dashed arrows. The epitope E-8 mapped in Chapter 3 is shown in bracket.


A5
E-8
100
A4
A3a
200
NH2
E-5b
E-1d
DEN
A3b
250
E-1c
50
E-5b
E-1d E-5b
E-7
E-1c
SLE?
YF?
E-7
150
300
DEN
JE
MVE
SLE
TBE
i2
350
400
B1
E-1d
E-5b
E-7
B4
COOH
450

domain A not previously identified as neutralization determinants. The E-7 epitope, which is separate from the other MVE epitopes by CBA, cannot be mapped with certainty; however if Ala 358 is a determinant, it is in a domain homologous to domain B.

The MVE E-1c epitope is defined by mAb with high specific activities in neutralization, HI and passive protection assays (Hawkes *et al.*, 1988). For TBE, this reactivity pattern seems to define mAbs against domain A epitopes; mAbs against these epitopes have the higher specific activities in neutralization or HI assays than mAbs against domains B and C (Guirakhoo *et al.*, 1989). In particular, mAbs against TBE epitopes A3 and A4 show strong activities in both neutralization and HI assays; the former also protects mice (Heinz *et al.*, 1983) and is therefore similar in properties to anti-E-1c mAb. An mAb against the SLE E-1c epitope also shows strong activities in neutralization, HI and protection assays but the location of this epitope has not been established (Roehrig, 1986). Neutralization determinants in YF (residues 71, 72) are also defined by mAbs with both neutralization and HI activities; however, passive protection studies with these mAbs have not been reported (see Lobigs *et al.*, 1987). For JE, residues 303-396 of E contain ten epitopes which are all associated with neutralization; mAbs against six of these epitopes passively protect mice, but none of the ten mAbs show HI activities (Mason *et al.*, 1989).

Considered together our data suggest that the MVE epitope E-1c is in a domain of the E protein which corresponds functionally and in terms of primary amino acid sequence to domain A. Domain A includes a highly conserved sequence (residues 98-111; Fig. 4.5) which is hydrophobic, glycine-rich and flanked by hydrophilic regions which are not conserved in sequence. This conserved region may be involved in an early, acid-induced fusion event during endocytosis. In support of this, epitopes in domain A of TBE undergo conformational changes at pH 6 (see above). Epitope E-1c of SLE and MVE are also sensitive to incubation at pH 6 as judged by reactivity with

the corresponding mAbs (J. T. R., personal communication). It is therefore possible that neutralization of flaviviruses by some mAbs specific for domain A occurs by inhibition of the acid-induced fusion. Such a mechanism has been proposed for WN (Gollins and Porterfield, 1986), herpes simplex virus (Fuller and Spear, 1987; Fuller *et al.*, 1989) and rabies virus (Dietzschold *et al.*, 1987).

Virulence of MVE antigenic variants

We have examined seven antigenic variants of MVE for evidence of attenuation following ip inoculation of 21-day old mice. In these variants there were various combinations of changes in E from the prototype sequence at residues 51, 126, 134, 263, 274, 276, 277 and 358. No variants were completely 'attenuated' according to the criteria of Monath *et al.* (1980). However variant P5a1 was of intermediate virulence (Table 4.2); variant P4a1 had an ic/ip LD50 ratio 10-fold lower than MVE-1-51 indicating a significant though small reduction in virulence. Thus two variants altered in the E-1c epitope (at residues 277 and 276 respectively) appeared to be attenuated. Other E-1c variants altered at residues 274 (Phe→Val) and 126 (Ala→Glu) did not appear to be attenuated. Thus attenuation did not correlate completely with resistance to an mAb directed against the E-1c epitope. The different effects on virulence of various changes in the E-1c epitope could have several interpretations. The first is that *any* change at residue 277 is more influential than *any* change at residue 276 (i.e. that amino acid position is of prime importance); the second is that the nature of the amino acid substitution is critical; the third possible explanation is that all changes at the E-1c epitope did lead to partial attenuation but that reversion to a virulent form occurred in the variants for which no attenuation was seen. These possibilities should be amenable to study with an infectious MVE cDNA clone, when available.

In earlier studies (Lobigs *et al.*, 1988), all seven isolates of MVE which were assessed for virulence following ip inoculation had a virulent phenotype; however, two showed a 'prozone' effect (see below). The seven isolates included MVE-1-51, four

others from Australia and two from Papua New Guinea. At the genetic level, the seven isolates differed in E protein sequence by up to eleven residues in pairwise comparisons (Lobigs *et al.*, 1988). The positions of difference from the prototype sequence were at residues 64, 123, 134, 141, 165, 166, 187, 229, 238, 240, 270, 275, 310 and 442. Since a 'prozone' effect could be regarded as evidence of attenuation, it is of interest to note the changes for isolates in which a 'prozone' effect was seen. The changes in MRM15019 were at residues 134 (Asp→Asn), 238 (Ile→Val) and 275 (Ser→Pro); the changes in MK6684 were at residues 123 (Asn→Ser), 134 (Asp→Asn), 141 (Val→Ile), 165 (Val→Ala), 229 (Ala→Ser), 240 (Val→Met) and 275 (Ser→Ala). It is of interest that nonconservative changes at residue 275 were observed in both variants.

Comparative sequence data exist for the E protein of the SA-14-14-2 vaccine strain of JE and the SA-14 parent strain (Nitayaphan *et al.*, 1990). There are five differences in E between these strains. These are at residues 107, 138, 176, 243 and 279. The change at residue 279 (Lys→Met) is nonconservative and close to residue 277 in MVE E protein (Lys 279 in JE corresponds to Lys 280 in MVE). This also supports the view that residues in the E-1c region are involved in an important function which may affect virulence.

The above data indicate that the E-1c region of domain A of the MVE E protein may have functions which determine the pattern of replication of the virus in vivo and its virulence. Data for MVE and other flaviviruses also suggest that epitopes in domain B determine virulence in mice. Lobigs *et al.* (1990) and A. Nestorowicz (unpublished results) have demonstrated that MVE passaged in tissue culture and changed in E at Asp 390 is completely attenuated in 21-day old mice (see above). Although it has not been rigorously demonstrated that the change at residue 390 causes attenuation, data showing that a single-step neutralization escape variant of TBE altered in the same region (Tyr 384→ His) is also attenuated supports the view that this region of domain B is also critical for pathogenesis. Comparative sequence data for the vaccine and the

parental strains of YF also suggest an important role for this region of domain B (Chapter 1).

In summary, we can define two regions of the MVE E protein which influence virulence in 21-day old mice. These are the residues around Ser 277 (in domain A) and Asp 390 (in domain B). It is not clear whether residues 277 and 390 are close in the tertiary structure of E; an answer to this point will be of importance in furthering our understanding of the molecular basis of virulence.

CHAPTER 5

EXPRESSION OF MURRAY VALLEY ENCEPHALITIS VIRUS STRUCTURAL PROTEINS IN INSECT CELLS USING A BACULOVIRUS VECTOR

5.1 INTRODUCTION

In Chapters 2 and 3, we investigated the expression of MVE cDNA in *E.coli*, with a view to generating MVE E protein fragments for analysis of functional determinants. Various fragments of E from 24 to 284 amino acids long were fused to ß-galactosidase. The fusion proteins reacted with anti-MVE HIAF in Western blots indicating that they carried MVE epitopes. A neutralizing mAb defining the E-8 epitope also reacted with E fusion proteins to allow mapping of this epitope to residues 201-224 in domain A. However four other neutralizing mAbs did not react with the E fusion proteins. The fusion proteins elicited antibodies against MVE in mice but these antibodies did not neutralize the virus. We concluded that the fusion proteins generated in *E.coli* could be useful in mapping certain epitopes, but that they were probably not suitable for a comprehensive analysis of the structure and function of E.

In this chapter, we explore the expression of MVE proteins in a baculovirus expression system. The baculovirus system was developed with the aim of generating large quantities of 'native' foreign protein (Summers and Smith, 1987) and has been shown to process eukaryotic proteins properly with respect to glycosylation, phosphorylation and proteolytic cleavage. Correct targeting of the foreign proteins to the cell surface, to nuclei or for secretion is observed in the *Spodoptera frugiperda* (Sf9) cell line used for such studies (Luckow and Summers, 1988a). Important features of the system include the strong polyhedrin promoter, the ease of propagating Sf9 cells and the efficient infection of Sf9 cells with baculoviruses (Summers and Smith, 1987). A range of plasmid 'transfer' vectors is available (Luckow and Summers, 1988b; 1989), among the most efficient is pVL941 which contains *Autographa californica* nuclear polyhedrosis virus (AcNPV) DNA sequences. These vactors allow homologous recombination with baculovirus DNA; the recombinant viruses can express open reading frames introduced into the transfer vector as non-fused proteins (Luckow and Summers, 1989).

Previous studies on the expression of flavivirus structural proteins in a baculovirus sytem have been with JE and DEN-4 (Maatsura *et al.*, 1989; Zhang *et al.*, 1988). The structural proteins of Sindbis virus, an alphavirus, have also been expressed in a baculovirus sytem (Oker-Blom and Summers, 1989). The studies with JE demonstrate that prM and E can be generated in Sf9 cells by proteolytic processing. These proteins were identified by their reactivities with protein specific mAbs and elicited neutralizing antibodies in mice. The studies with DEN-4 showed that prM and E were generated and on immunization protect mice against ic challenge with lethal doses of DEN-4.

In this chapter we introduce, via pVL941, genes for the MVE proteins C, prM and E into the AcNPV genome. We examine the production, proteolytic cleavage, glycosylation, yield and conformation of the expressed proteins.

5.2 MATERIALS AND METHODS

5.2.1 Cells and viruses

Spodoptera frugiperda cells (clone Sf9) were obtained from the American Type Culture Collection (ATCC; Rockville, Maryland, USA) and were propagated at 27° in TNM-FH medium plus 10% FCS (Summers and Smith, 1987). TNM-FH was Grace medium (Grace, 1962) supplemented with 6.6 g/litre lactalbumin hydrolysate (Difco) and 6.6 g/litre yeastolate (Difco). SW13, a human adenocarcinoma cell line, was grown at 37° in EMEM supplemented with 10% FCS. *Aedes albopictus* cells (C6/36; Igarashi, 1978) were grown at 28° in Eagle's basal medium (EBM) containing 10% FCS.

Autographa californica nuclear polyhedrosis virus (AcNPV) was a gift from Dr M.-C. Fung (Division of Clinical Sciences, John Curtin School of Medical Research, Australian National University). MVE-1-51 was used as a 10% suckling mouse brain homogenate in HBSS. Purified MVE was provided by Dr R. C. Weir (Materials and Methods, Chapter 3).

5.2.2 Anti-MVE HIAF and anti-E mAbs

See Materials and Methods, Chapter 3.

5.2.3 Plasmids, plasmid preparation, restriction enzyme digestion, transformation of *E.coli*

Procedures were as described for Chapter 2 or Chapter 3. The baculovirus transfer vector pVL941 (9.8 kb; Luckow and Summers, 1989) was a gift from Dr M.-C. Fung. It contains 7.8 kb of AcNPV genome sequence, the pUC8 plasmid and codes for ampicillin resistance (see also Fig. 5.1).

5.2.4 Generation of recombinant MVE-baculovirus tranfer vector

Preparation of vector DNA: pVL941 was used to transform *E.coli* MC1061.1 with ampicillin selection (100 µg/ml ampicillin in LB agar). Plasmid DNA was extracted by alkaline lysis and quantitated by UV spectrophotometry. DNA (5 µg) was incubated with BamHI (5 units) at 37° for 1 hour. Calf intestinal alkaline phosphatase (0.2 units) was added and the mixture incubated at 37° for 30 min and at 70° for 10 min. DNA was extracted with phenol and quantitated by ethidium bromide staining.

Ligation of BamHI linkers to MVE cDNA: MVE cDNA (p2/1/22; encoding the 5' 5.4 kb of the MVE genome) was digested with EcoRI and SacI (5 units each), blunt-ended with T4 DNA polymerase and purified on LMT agarose. BamHI linkers (5 µg; CGGGATCCCG; New England Biolabs) were phosphorylated by incubation with 0.4 mM ATP, 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT and T4 polynucleotide kinase (9 units; BRL, Life Technologies Inc., Gaithersburg, USA) at 37° for 1 hour and the reaction stopped by heating at 90° for 5 min. The 2.5 kb MVE cDNA fragment (0.5 µg; see Fig. 5.2) was mixed with the phosphorylated BamHI linkers (0.2 µg) and incubated with T4 DNA ligase (100 units, New England Biolabs) at room temperature overnight. BamHI (50 units) was added; incubation was at 37° for

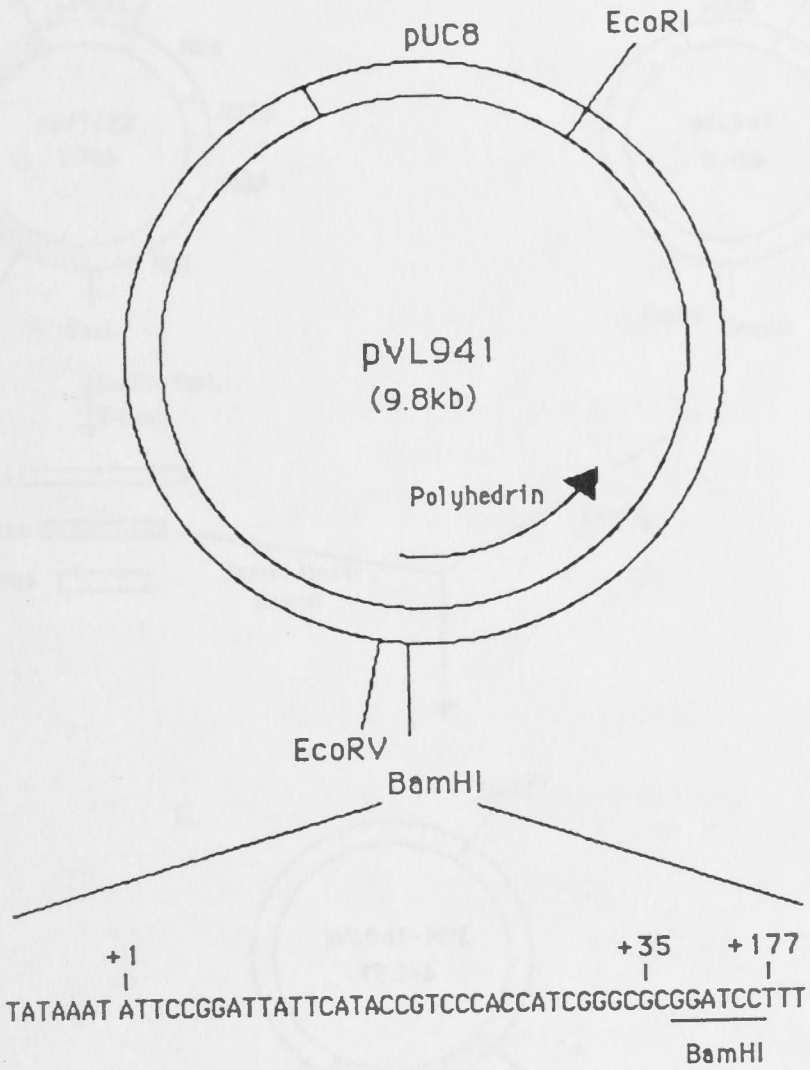


Figure 5.1 The transfer vector pVL941 (Summers and Smith, 1987; Luckow and Summers, 1989). pVL941 (9.8 kb) consists of pUC8 (2.7 kb) and a fragment (7.1 kb) from the genome of AcNPV containing the polyhedrin gene (≈0.7 kb; solid arrow) and flanking sequences. A unique BamH1 site is at position +171 in the polyhedrin coding region (+1 represents the first nucleotide of the polyhedrin coding region). A segment of the polyhedrin gene from +36 to +170 is deleted. The ATG initiation codon of the coding sequence is mutated to ATT for expression of nonfused recombinant proteins. The nucleotide sequence of the polyhedrin gene is shown from position -7 to +179. The unique EcoRI and EcoRV sites are indicated.

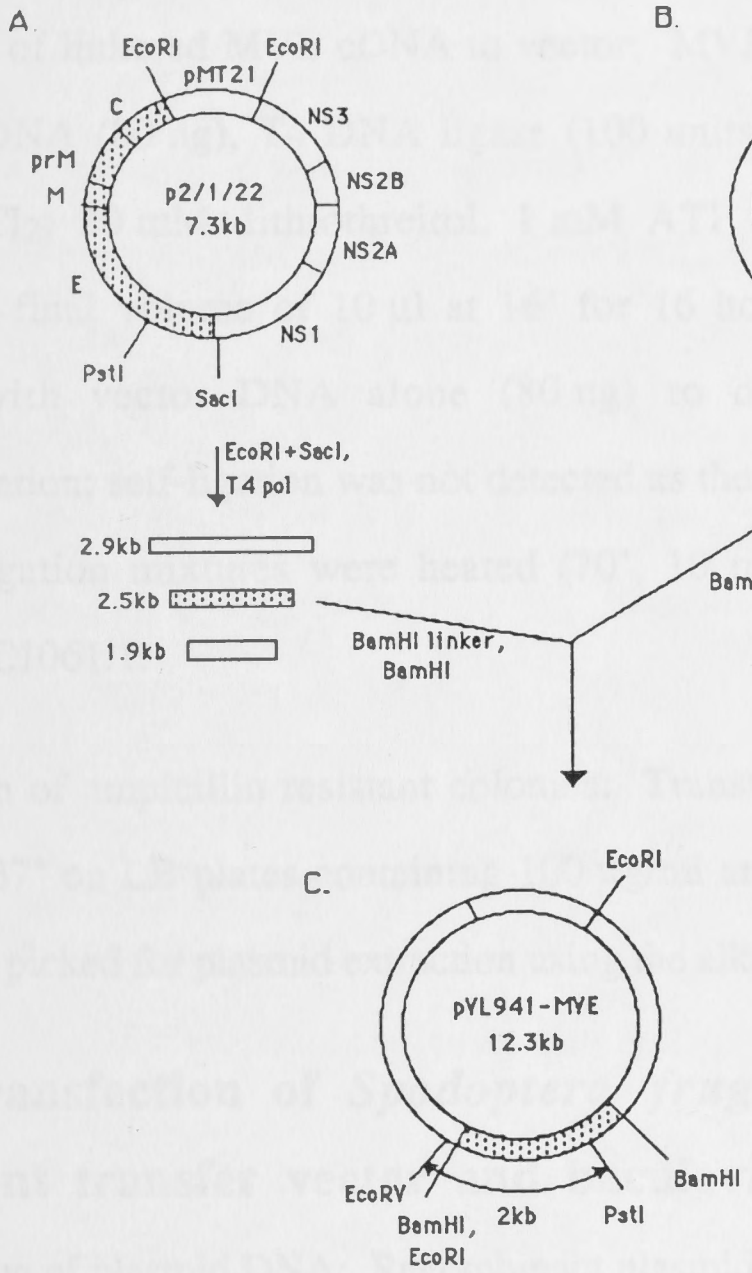


Figure 5.2 Construction of the pVL941-MVE recombinant plasmid. (A) Plasmid p2/1/22 (7.3 kb) contains 5' non-coding regions (≈90 nucleotides) and coding regions for MVE C, prM, E NS1, NS2A, NS2B and half of NS3. Incubation of p2/1/22 (5 µg) with EcoRI and SacI (5 units each) produced three fragments (2.9 kb, 2.5 kb, 1.9 kb), which were blunt-ended with T4 DNA polymerase and separated on 1% LMT agarose. The 2.5 kb fragment contains the 5' untranslated region, the structural proteins (C, prM, E) and part of NS1 (11 amino acids). It was isolated and BamHI cohesive ends were attached before ligation to BamHI-digested, dephosphorylated pVL941 (B). The resulting pVL941-MVE plasmid (C) was 12.3 kb in size and contained a new BamHI restriction fragment (2.5 kb) and an EcoRV-PstI restriction fragment (2 kb).

4 hours to produce BamHI cohesive ends and to remove concatemers of linkers from MVE cDNA. Linkered MVE cDNA was extracted from 1% LMT agarose and quantitated on the basis of ethidium bromide staining.

Ligation of linkered MVE cDNA to vector: MVE cDNA (5 ng) was incubated with vector DNA (80 ng), T4 DNA ligase (100 units), 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 20 mM dithiothreitol, 1 mM ATP and 50 µg/ml bovine serum albumin in a final volume of 10 µl at 16° for 16 hours. A control ligation was performed with vector DNA alone (80 ng) to determine the efficiency of dephosphorylation; self-ligation was not detected as there were no ampicillin-resistant colonies. Ligation mixtures were heated (70°, 10 min) before transformation of competent MC1061.1.

Selection of ampicillin resistant colonies: Transformed MC1061.1 was grown overnight at 37° on LB plates containing 100 µg/ml ampicillin. Ampicillin resistant colonies were picked for plasmid extraction using the alkaline lysis method.

5.2.5 Cotransfection of *Spodoptera frugiperda* cells with recombinant transfer vector and baculovirus DNA

Extraction of plasmid DNA: Recombinant plasmid (pVL941-MVE) was extracted by alkaline lysis and further purified by addition of SDS to 0.5% and proteinase K (Boehringer) to 20 µg/ml, followed by incubation at 50° for 1 hour. DNA was extracted with phenol/chloroform, chloroform and twice with diethyl ether (Ajax). Traces of ether were removed by incubation at 70° for 5 min in an open Eppendorf tube. Plasmid DNA was precipitated with 7.5 M ammonium acetate (1/2 volume) and ethanol (2 volumes), held on dry ice for 30 min and pelleted by centrifugation. DNA pellets were washed once with 80% ethanol and resuspended in 10 mM TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5).

Preparation of baculovirus genome DNA (Summers and Smith, 1987): Sf9 cell monolayers (3×10^7 cells in 75 cm^2 flasks; Corning) were infected with AcNPV (MOI≈1). Supernatant was collected at 3 days p.i. and centrifuged at 2500 rpm for 10 min. The virus was pelleted by centrifugation for 30 min at 24,000 rpm. The pellet was resuspended in 0.1 x TE, layered on a linear 25-56% sucrose gradient in 0.1 x TE (11 ml in SW41 tubes) and centrifuged at 18,000 rpm for 90 min. The viral band (3 ml) was collected, diluted with 0.1 x TE (5 ml) and pelleted in an SW41 tube (18,000 rpm, 30 min). The pellet was resuspended in 4.5 ml extraction buffer (0.1 M Tris-HCl, pH 7.5, 0.2 M KCl, 0.09 M Na_2EDTA) containing 0.2 mg of proteinase K and incubated for 1 hour at 50°. Sarkosyl (Sigma; 0.25 ml of 20% solution) was added for a further 2 hours at 50°. The mixture was extracted twice with a phenol:isoamyl alcohol:chloroform mixture (25:1:24; 5 ml) and the phases separated by centrifugation at 2000 rpm (Sorvall SS-34 rotor). Ethanol (10 ml) was added and DNA precipitated (-80°, 10 min). The pellet was collected by centrifugation at 2500 rpm for 20 min, washed with ethanol (90%) and resuspended in 0.1 x TE (0.5 ml). Incubation at 65° for 15 min and 4° overnight was required to dissolve the DNA which was stored at 4°.

Cotransfection of Sf9 cells (Summers and Smith, 1987): Sf9 cells were incubated to form monolayers in 25 cm^2 flasks (Corning). The TNM-FH medium was replaced with Grace medium (0.75 ml) containing 10% FCS. Cotransfection was with 2×10^6 Sf9 cells using 1 µg of AcNPV DNA and 4 µg of pVL941-MVE DNA. The DNA mixture in transfection buffer (0.75 ml; 0.025 M HEPES, pH 7.1, 0.14 M NaCl, 0.125 M $CaCl_2$) was added drop-wise. The medium was removed after incubation for 4 hours at 27° and replaced with TNM-FH containing 10% FCS. Cells were incubated for four days at 27°. The supernatant containing a mixture of wild-type and recombinant AcNPV was harvested and stored at 4°.

5.2.6 Screening procedures and isolation of recombinant MVE-baculovirus

Three methods were tested. These involved an assessment of plaque morphology, reactivity with HIAF against MVE or hybridization with MVE cDNA.

Method 1 (Summers and Smith, 1987): Recombinant AcNPV does not produce the polyhedrin protein which is the major component of the virus-containing occlusion bodies in the nuclei. Thus, normal AcNPV plaques consist of cells with enlarged nuclei containing occlusion bodies, while recombinant plaques show enlarged nuclei without occlusion bodies. Both wild-type and recombinant plaques are visible by eye in unstained plates. However in order to distinguish wild-type from recombinant plaques (which do not form occlusion bodies), light microscopy is needed. The titre of the mixture of AcNPV and recombinant virus in the cotransfection supernatant was determined by plaque formation on Sf9 cell monolayers. Virus (1 ml) diluted in TNM-FH was used to infect semi-confluent monolayers of Sf9 cells (2×10^6) in 60 mm Petri dishes (Nunc, Roskilde, Denmark) by incubation at 27° for 1 hour with frequent rocking. The inoculum was removed and monolayers overlaid with TNM-FH containing 1.5% (w/v) LMT agarose (Seaplaque, Rockland, USA) and 10% FCS. Incubation was for 4 days at 27° followed by light microscopy.

Method 2: This was based on performing limiting dilutions of the cotransfection supernatant and the immunodetection of MVE antigens in infected Sf9 cells. The cotransfection supernatant was used to infect Sf9 cells (1.5×10^5 per well) in 96-well flat-bottom trays (Nunc) in dilutions from 10^{-2} to 10^{-6}. After incubation for 4 to 6 days, the cells were lysed in radio-immunoprecipitation (RIP) buffer, a solution containing 0.15 M NaCl, 10 mM EDTA, 1% sodium deoxycholate, 1% Nonidet P-40 (NP-40; Sigma), 0.1% Triton X-100 and 20 mM Tris-HCl (pH 7.8). The mixture was transferred to nitrocellulose and probed with anti-MVE HIAF (Chapter 2).

Method 3 (adapted from Fung *et al.*, 1987): The cotransfection supernatant was diluted from 10^{-2} to 10^{-6} and 40 µl aliquots used to infect Sf9 cells in 96-well trays (as above). After 6 to 8 days at 27°, the supernatant in each well was transferred to a duplicate 96-well tray for storage at 4°. The Sf9 cells were lysed in 0.2 M NaOH (0.2 ml) and incubated (15 min, room temperature). Lysates from each well were transferred to nitrocellulose pre-equilibrated with 20 X SSC (1 X SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.4) in a Bio-Dot apparatus (Bio Rad). 2 M NaCl (0.4 ml) was added to each well before the lysates and suction applied. The filter was washed once in 2 X SSC and baked (2 hours, 80°) under vacuum in a slab-gel dryer (Bio-Rad). It was then incubated (3 hours, 68°) in pre-hybridization fluid: 1.5 X SSPE (0.27 M NaCl, 0.015 M EDTA, 0.015 M NaH_2PO_4, pH 7.5), 1% SDS and 0.5% BLOTTO (10% non-fat milk powder in 0.2% NaN_3). A ^{32}P-labelled DNA probe was generated from p2/1/22 by nick-translation. p2/1/22 (0.5 µg) was incubated with DNase1 (1 ng; bovine pancreatic; Sigma) for 20 min at 14° in the presence of 50 mM Tris-HCl (pH 7.5), 7.5 mM magnesium acetate, 5.7 mM DTT, 0.1 mg/ml bovine serum albumin (BRL), 2.86 µmoles each of dATP, dGTP, dTTP and 6.6 pmoles of α-^{32}P-dCTP (3 Ci/µmole); the final volume was 35 µl. *E.coli* DNA polymerase 1 (2.5 units; New England Biolabs) was added (20 min, 14°). EDTA was added to 30 mM to terminate nick-translation. Immediately prior to hybridization, 0.3 ml of 30 mM NaOH was added to the probe (40 µl), the mixture was incubated for 5 min in a boiling water bath and quick-chilled. Denatured probe (0.34 ml) was added to 5 ml of freshly prepared pre-hybridization fluid and incubated with the nitrocellulose membrane for 15 hours at 68° (shaking was not necessary). The membrane was washed sequentially in (1) 2 X SSC, 0.1% SDS; (2) 0.5 X SSC, 0.1% SDS and (3) 0.1 X SSC, 0.1% SDS for 15 min each at room temperature, then in 0.1 X SSC, 1% SDS for 30 min at 50°. The air-dried nitrocellulose was exposed to Fuji X-ray film for autoradiography.

5.2.7 Extraction of nuclear DNA from infected *Spodoptera frugiperda* cells (Summers and Smith, 1987)

Sf9 cell monolayers (2 x 10^6 cells) in 25 cm^2 flasks were infected with wild-type or recombinant AcNPV (MOI≈0.1) and incubated for 4 days. The supernatant was removed and the cells resuspended in 5 ml of lysis buffer (10 mM magnesium acetate, 1% NP-40, 30 mM Tris-HCl, pH 7.5). Incubation was for 3 min at room temperature and 5 min at 4°. Nuclei were collected by centrifugation at 2000 rpm for 3 min, washed once with PBS, re-pelleted and resuspended in extraction buffer (4.75 ml) containing 0.2 mg of proteinase K. Purification of DNA was as in Section 5.2.5. DNA was resuspended in 0.5 ml 0.1 x TE and stored at 4°.

5.2.8 Semi-quantitative comparison of polyhedrin mRNAs by dot-blot hybridization (Luckow and Summers, 1988b)

Sf9 cells (5 x 10^6) in 25 cm^2 flasks were infected with wild-type or recombinant AcNPV (MOI≈10) and incubated for 2 days at 27°. The cells were dislodged by shaking, collected by centrifugation at 2000 rpm for 5 min and washed once in ice-cold Grace medium. The cell pellet was resuspended in 45 µl of ice-cold 1 x TE containing 5 µl of 5% NP-40 and incubated for 5 min at 4°. Another 5 µl of NP-40 was added and nuclei pelleted by centrifugation for 2.5 min in an Eppendorf microfuge at 4°. The supernatant (50 µl) was incubated with 20 X SSC (30 µl) and formaldehyde (20 µl; 37% solution; Sigma) for 15 min at 65° and stored at -80°. The crude cytoplasmic extract was used for hybridization without further treatment. It was diluted serially with 15 X SSC. Aliquots (100 µl) equivalent to 2 x 10^3, 10^4, 5 x 10^4 and 2.5 x 10^5 cells were dot-blotted on nitrocellulose, rinsed with 6 X SSC and baked for 2 hours at 80° under vacuum. Hybridization probes were α-^{32}P-labelled pVL941 and α-^{32}P-labelled p2/1/22 (see above). Levels of polyhedrin mRNAs were estimated from hybridization signals with the pVL941 probe. It was expected that the pVL941 probe would be of low mol. wt. (<400 bp) and not affected by the presence of additional

sequence in the coding regions of the polyhedrin and polyhedrin-MVE mRNAs. We have assumed that it hybridizes to these mRNAs with approximately equal efficiency.

5.2.9 SDS-PAGE and immunoblot analysis of infected cell lysates

Sf9 cell monolayers in 24-well trays (Nunc) were infected with wild-type or recombinant AcNPV (MOI≈40), incubated for 48 hours and harvested in 100 µl of ice-cold RIP buffer. Cells were agitated for 10 min to completely lyse cells. Debris was removed by centrifugation for 2 min and clarified lysates stored at -20°. Aliquots (10 µl) equivalent to 5×10^4 cells were made up to 16 µl in SDS-PAGE sample buffer (Laemmli, 1970) containing 1% SDS and 5% ß-ME (reducing condition) or in identical sample buffer containing 0.1% SDS and no ß-ME (non-reducing condition; Cohen *et al.*, 1986). Samples containing ß-ME were held in boiling water for 2 min. Electrophoresis was on SDS-20% PA gels for both reduced and unreduced samples. As controls, purified MVE and MVE-infected Vero and C6/36 cell lysates were used. Proteins were stained in gels with Coomassie Brilliant Blue or electrophoretically transferred to nitrocellulose for detection using anti-MVE HIAF and mAbs (Chapter 3.). As size standards, low mol. wt. proteins (14.4-94K; Pharmacia) were electrophoresed and transferred to nitrocellulose for staining in 0.1% (v/v) 'India' ink (Rotring, Germany) in PBS and 0.3% Tween-20.

5.2.10 Radiolabelling, immunoprecipitation and fluorography of proteins in infected cells

Sf9 cell monolayers in 24-well trays were infected with wild-type or recombinant AcNPV (MOI≈40) and incubated at 27°. At 47 hours p.i., the medium was replaced with Grace medium (1 ml) lacking methionine; ^{35}S-methionine (80 µCi/ml final concentration; 800 Ci/mmole; Amersham) was added for 10 min at 48 hours p.i.. After 10 min, labelling medium was replaced with Grace medium containing a 20-fold excess of 'cold' methionine plus 0.1 mg/ml cycloheximide (Boehringer) and incubated for 1.5

or 3 hours (see text). Cells were harvested in RIP buffer (100 µl) before or after the chase. Where indicated, tunicamycin (10 µg/ml; Boehringer) was added 4 hours before labelling. C6/36 cells were infected with MVE-1-51 (MOI≈10) and incubated at 28° in EMEM containing 10% FCS. At 20 hours p.i., medium was replaced with EMEM lacking methionine. ^{35}S-methionine (30 µCi/ml) was added at 21 hours p.i. and lysates harvested in RIP buffer after incubation for 2 hours.

For immunoprecipitation, labelled lysates (10 µl) were diluted in 0.1 ml RIP buffer containing 1-2 µl of undiluted anti-MVE HIAF and incubated at 4° overnight. *Staphylococcus aureus* (Cowan I strain) cells (Calbiochem, Behring Diagnostics, La Jolla, California, USA) were added (40 µl of a 10% suspension in RIP buffer) to the mixture; incubation was at 4° for 1 hour. Cells were collected by centrifugation (4000 rpm, 15 min) through 10% sucrose in RIP buffer (1 ml). Cells were washed twice with RIP buffer, resuspended in 20 µl of SDS-PAGE sample buffer and incubated for 30 min at 65°. After centrifugation for 2 min, the supernatant was analysed by SDS-PAGE (Chapter 2). For fluorography, gels were stained with Coomassie Brilliant Blue, destained, soaked for 15 min in Amplify (20 ml; Amersham) and dried at 80° in a slab gel dryer (Bio-Rad). Fuji RX100 X-ray film was used for fluorography. Low mol. wt. marker proteins (14.4K-94K) were electrophoresed and stained on each gel.

5.2.11 Localization of MVE E protein by immuno-fluorescence (Harlow and Lane, 1988; Hardham, 1985)

To determine the intracellular distribution of MVE E protein, fixation of cells was in paraformaldehyde (Harlow and Lane, 1988). To demonstrate the existence of E on cell surface, paraformaldehyde-glutaraldehyde fixation was used (Hardham, 1985). Sf9 cells on glass coverslips in 35 mm Petri dishes (Nunc) were infected in duplicate with wild-type or recombinant AcNPV (MOI≈10). As controls C6/36 cells were infected with MVE (MOI≈10) in duplicate or were mock-infected. At 48 hours p.i., coverslips were washed in PBS and either fixed in 4% paraformaldehyde (Sigma) for 10 min for

detection of internal antigens, or in 4% paraformaldehyde-0.2% glutaraldehyde (Aldrich) for 1 hour for detection of cell surface antigens. Paraformaldehyde was freshly prepared in PBS (Harlow and Lane, 1988). For detection of intracellular antigens, cells on coverslips were permeabilized for 3 min in PBS containing 0.2% Triton X-100; washed twice in PBS and incubated for 10 min in blocking solution containing PBS, 5% (w/v) milk powder and 5% FCS. To detect cell surface antigens, fixed cells were rinsed twice in a solution of buffered glycine (50 mM in PBS, pH 7.5) and incubated in blocking solution as above. The neutralizing mAb against the MVE E-5b epitope (see Table 3.1) reacts specifically with the unreduced E protein (Chapter 3) and was used for detection of E. Coverslips were incubated (1 hour, 37°) with anti-E-5b mAb diluted 10^{-3} with the blocking solution, washed in blocking solution (x 3) and incubated (30 min, 37°) with FITC-conjugated anti-(mouse IgG) (Bio-Yeda Ltd., Kiryat Weizmann, Rehovot, Israel) diluted 10^{-2} with blocking solution. Coverslips were mounted in a solution of glycerol (30%) in PBS and immediately examined by UV microscopy. Filter settings were appropriate for FITC fluorescence; oil-immersion lenses (40X or 100X; Olympus) were used. Photographs were taken with the assistance of Dr A. Hardham (Research School of Biological Sciences, Australian National University) using Kodak films (Tri-X or T-max; SA400) rated at ASA1600.

5.3 RESULTS

5.3.1 Construction of recombinant pVL941 transfer vector

The first step in the expression of the MVE structural proteins was to construct a transfer vector (pVL941) with the appropriate MVE cDNA inserted downstream of the polyhedrin promoter. pVL941 (9.8 kb) has a unique BamHI site 37 bp downstream from a mutated, inactive, polyhedrin initiation codon and a polyhedrin coding region with a deletion between nucleotides 36 and 170 (Fig. 5.1). The MVE cDNA clone used for the construction is shown in Fig. 5.2A. p2/1/22 is 7.3 kb and consists of pMT21 with 5.4 kb of MVE cDNA introduced at the unique EcoRI site. The insert codes for

almost the complete 5' non-coding region (≈90 nucleotides), C, prM, E, NS1, NS2A, NS2B and half of NS3 (Dalgarno *et al.*, 1986). By digesting with EcoRI and SacI, a cDNA fragment coding for the 5' untranslated region, C, prM, and E should be generated; it would also include 34 bp from the 5'-terminus of the NS1 gene (Fig. 5.2A).

Assuming successful construction, the recombinant baculovirus (see below) would generate a hybrid mRNA transcribed from the polyhedrin promoter 5' to the +1 nucleotide (Fig. 5.1). The transcript would terminate near the poly-A addition site of the polyhedrin mRNA (Summers and Smith, 1987). Translation would be expected to start at the C protein initiation triplet rather than at the mutated polyhedrin triplet (Fig. 5.1) and should generate a polyprotein containing C, prM, E, NS1 (11 amino acids) and part of the polyhedrin protein. The construct is deleted in the polyhedrin ORF from nucleotides 36-170 (Fig. 5.1); however readthrough past nucleotide 171 is arrested by a termination codon about 60 nucleotides downstream and the polyprotein would be expected to contain only 20 amino acids read from the polyhedrin mRNA.

To construct the recombinant plasmid, p2/1/22 was digested with EcoRI and SacI (Fig. 5.2). Fragments of 2.9 kb, 2.5 kb and 1.9 kb were produced as expected (Fig. 5.2A). They were blunt-ended with T4 DNA polymerase and the 2.5 kb fragment isolated. BamHI linkers were ligated to it and the mixture was digested with BamHI to produce a 2.5 kb fragment for ligation to BamHI-digested pVL941 vector (9.8 kb). The ligation mixture was used to transform *E.coli* MC1061.1. Twelve ampicillin resistant transformants were picked, plasmids were extracted, incubated with BamHI and electrophoresed on agarose gels. Two plasmids contained inserts of 2.5 kb at the BamHI site; the others contained no inserts. The two plasmids (12.3 kb) were incubated with PstI and EcoRV to determine the orientation of the MVE cDNA. The appropriate plasmid was expected to generate two fragments (10.3 kb, 2 kb) on incubation with PstI and EcoRV (Fig. 5.2C). One plasmid contained the MVE cDNA in

the reverse orientation. The other contained the expected BamHI and EcoRV-PstI fragments (Fig. 5.3) and is referred to below as pVL941-MVE.

5.3.2 Generation and isolation of MVE-baculovirus recombinant

Cotransfection of Sf9 cells with AcNPV genomic DNA and recombinant pVL941 generates wild-type virus and 0.1-10% of the recombinant baculovirus (Summers and Smith, 1987). Three methods were tested for the isolation of recombinant AcNPV-MVE from the cotransfection supernatant (see Materials and Methods for details). These relied on (1) the predicted distinct plaque morphology of AcNPV-MVE, (2) the detection of MVE proteins using anti-MVE HIAF, and (3) the detection of MVE cDNA sequences.

The first method was unsuccessful as none of the many plaques examined microscopically (≈100) were devoid of occlusion bodies, indicating that these cells were infected with the wild-type AcNPV and that the cotransfection supernatant contained a low percentage of recombinant virus. The second method was unsuccessful because weak signals were obtained and could not be differentiated from the background. The third method was successful. 10^{-2} to 10^{-6} dilutions of cotransfection supernatant were used to infect Sf9 cells in 96-well trays. After incubation for 6 days, supernatants were transferred to a fresh tray for storage. The cells in each well were lysed in sodium hydroxide and dot-blotted on nitrocellulose for detection of MVE sequences by hybridization with p2/1/22. Cells from nine out of twelve wells infected with a 10^{-2} dilution hybridized to MVE cDNA (the faint signal in well J was not reproduced adequately by photography in Fig. 5.4); at 10^{-3} cells from nine out of twelve wells hybridized, and at 10^{-4} cells from two out of twelve wells hybridized (Fig. 5.4). From the titre of the undiluted virus (10^8 PFU/ml) and the volume added, it was estimated that at 10^{-3} and 10^{-4} dilutions, each well was infected with approximately 4000 and 400 PFU of AcNPV respectively. Hence the positive wells (C, E) at a 10^{-4} dilution were

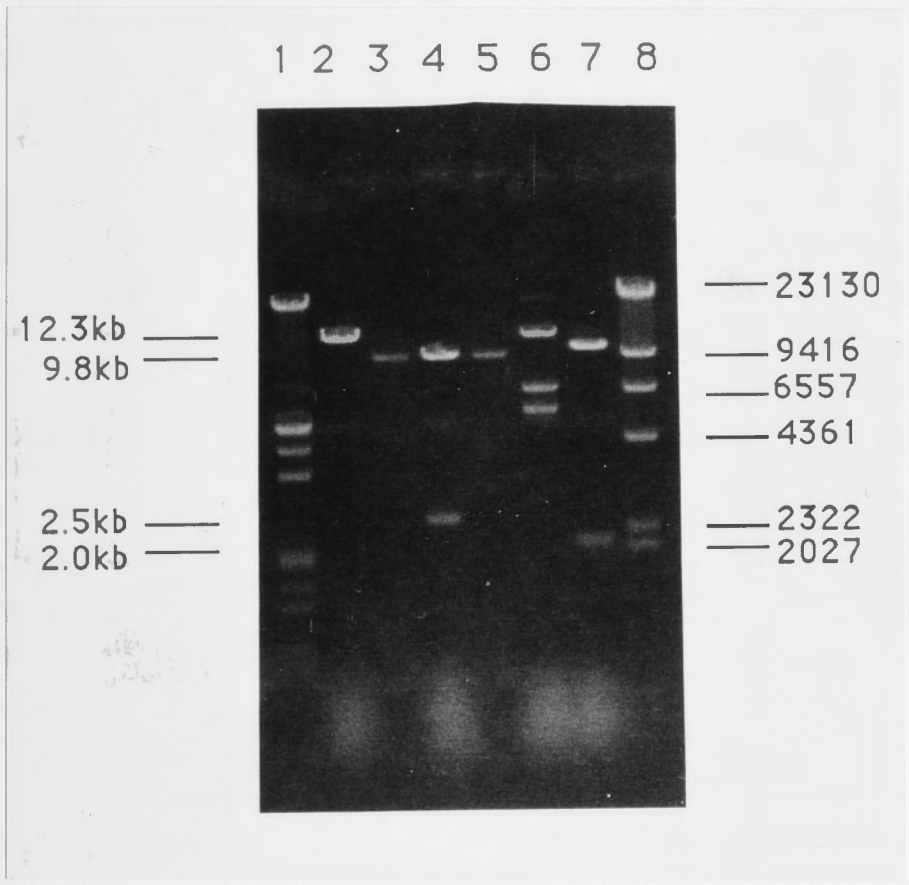


Figure 5.3 Restriction enzyme digestion of pVL941-MVE recombinant plasmid DNA. pVL941 DNA (Lanes 3, 5; 1 μg) and recombinant DNA (Lanes 2, 4, 6, 7; 1 μg) was incubated with 2 units each of PstI (Lane 2), BamHI (Lanes 3, 4), EcoRI (Lanes 5, 6), or EcoRV+ PstI (Lane 7) at 37° for 1 hour. Electrophoresis was on 1% agarose at 60 volts for 1.5 hours. The gel was stained in ethidium bromide (0.5 μg/ml) for 30 min, destained and photographed under UV. Size standards were λ DNA digested with HindIII and EcoRI (Lane 1) or HindIII alone (Lane 8).

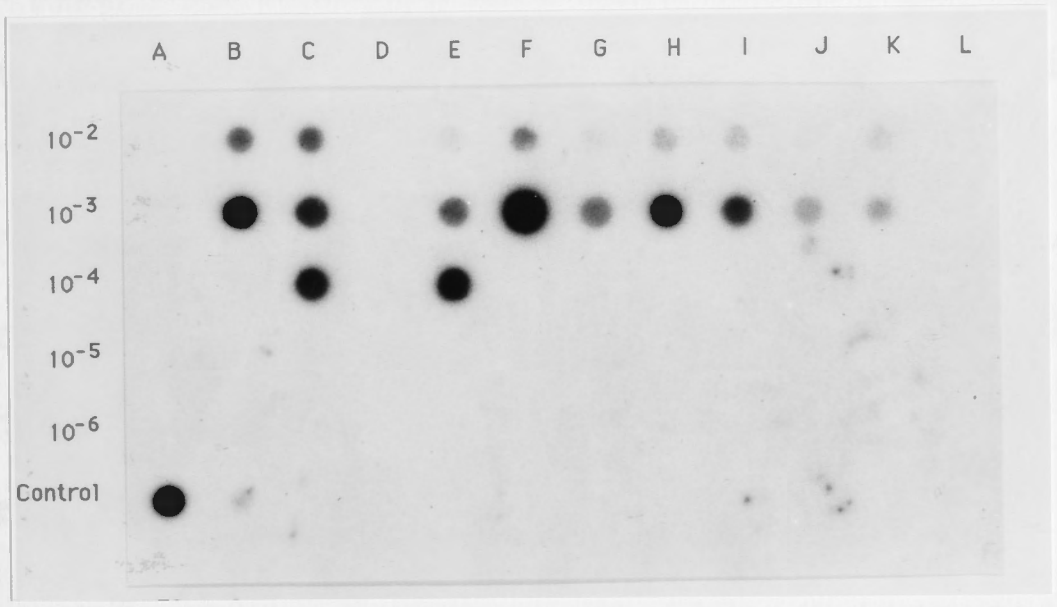


Figure 5.4 Hybridization screening for recombinant AcNPV. Sf9 cells in a 96-well tray (1×10^4 cells/well) were infected with 40 µl aliquots of diluted cotransfection supernatant. 12 wells (A to L) were infected for each dilution. Incubation was for 6 days at 28°. Lysate from each well was transferred to nitrocellulose (70 mm x 105 mm). Detection of MVE-specific sequences was by hybridization using the ^{32}P-labelled p2/1/22 and autoradiography. The controls (bottom left corner) were, from left to right, 30 ng, 0.1 ng of p2/1/22 plasmid DNA and DNA from AcNPV-infected Sf9 cells.

expected to be enriched in recombinant AcNPV by comparison with the positive wells at the 10^{-2} and 10^{-3} dilutions. The AcNPV-MVE recombinant was estimated to be less than 0.1% of the original cotransfection supernatant based on these results. Supernatants from the two positive wells (C, E; 10^{-4} dilution) were diluted 10^{-5} and 10^{-7} and used to infect 96-well trays (16 wells for 10^{-5} dilution, two trays for 10^{-7} dilution) for hybridization screening. Three positive wells were obtained with the 10^{-7} dilution. The supernatants were used to infect Sf9 cells in 25 cm^2 flasks. At 4 days p.i., enlarged nuclei were visible in most of the cells examined (≈500), but no occlusion bodies were detected indicating that the inoculum did not contain contaminating wild-type AcNPV. Virus was harvested at 4 days; titres were ≈$5x10^8$ PFU/ml (assayed by plaque titration). Three recombinant AcNPV stocks (Rec1, Rec2, Rec3) were obtained from two further rounds of screening. The latter two were derived from the same well (E) from the first round while Rec1 was from a different well (C) and thus was likely to be due to a different recombination event.

5.3.3 Restriction enzyme analysis of DNA from infected *Spodoptera frugiperda* cells

Rec1 and Rec2 were expected to contain 2.5 kb of the MVE cDNA in the polyhedrin coding region. This could be confirmed by comparing the restriction enzyme (RE) profiles of Rec1, Rec2 and wild-type AcNPV genome DNAs using BamHI, BglII and PstI. Fig. 5.5 shows that the polyhedrin gene is in the 3rd largest BglII fragment (≈13 kb) and the 4th largest PstI fragment (≈11 kb); it also contains a BamHI site where the MVE cDNA was inserted. Insertion of the MVE cDNA in the polyhedrin coding region was expected to alter these fragments and create a new BamHI fragment of 2.5 kb.

For RE analysis, total nuclear DNA from infected Sf9 cells was used, as 25% is AcNPV genomic DNA (Summers and Smith, 1987). Sf9 cells were infected with recombinant (Rec1 or Rec2) or with wild-type virus (MOI≈1) and nuclear DNA

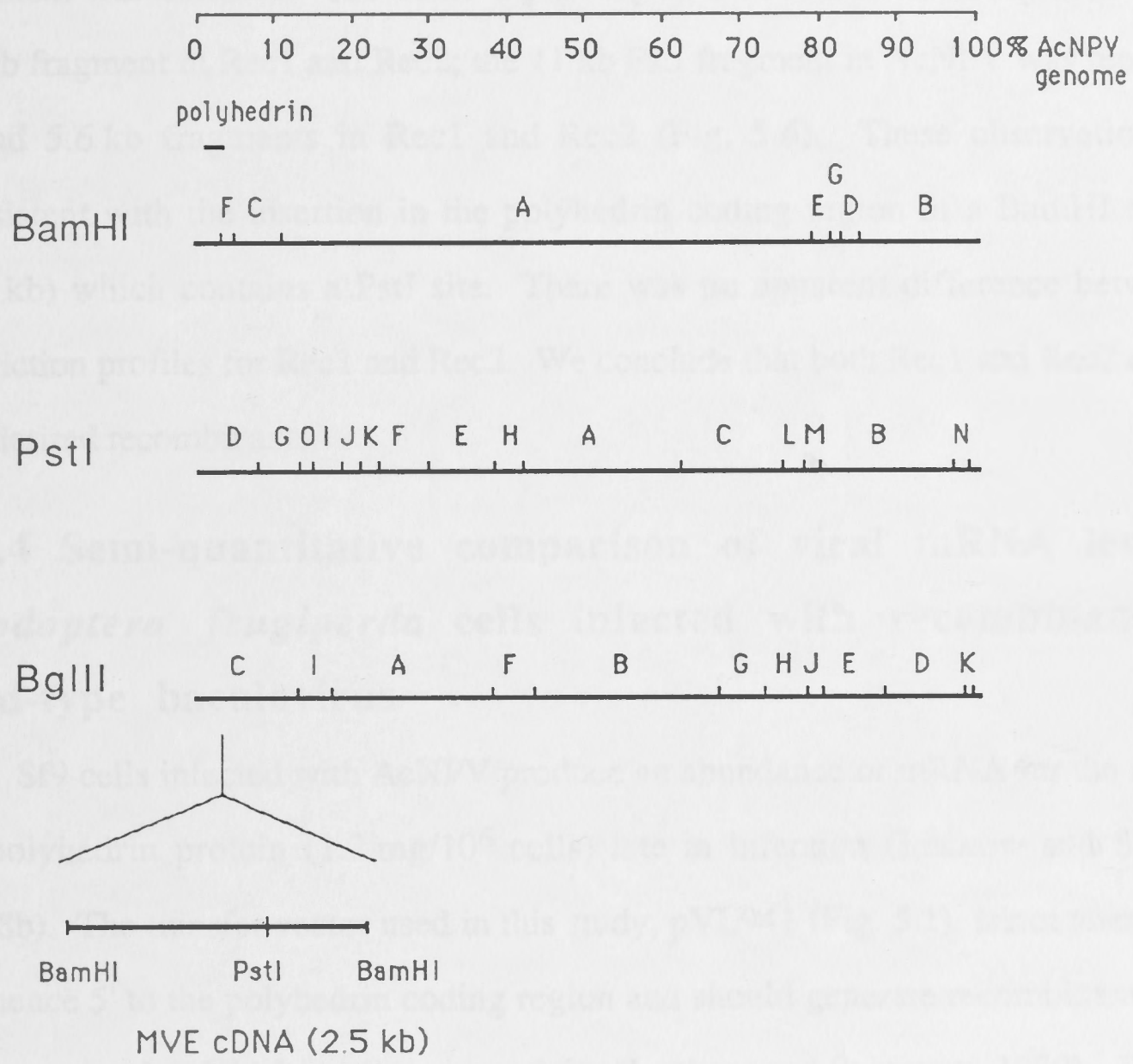


Figure 5.5 Restriction map of the AcNPV genome showing BamHI, PstI and BglII sites (Summers and Smith, 1987). The circular genome (≈120 kb) is linearized and is shown as solid lines. The largest fragment for each restriction enzyme is designated 'A', the second largest 'B' and so on. The coding region for the polyhedrin protein is indicated. The MVE cDNA fragment at the BamHI site in the polyhedrin gene is shown. It contains a PstI site but no BglII sites.

extracted at 3 days p.i. DNA from 10^5 cells was digested with BamHI, BglII or PstI and electrophoresed on 1% agarose (Fig. 5.6). Restriction fragments from recombinant and wild-type viral genomes were visible. With Rec1 and Rec2, a new 2.5 kb BamHI fragment was detected. The 13 kb BglII fragment in AcNPV was replaced by a 15-16 kb fragment in Rec1 and Rec2; the 11 kb PstI fragment in AcNPV was replaced by 8 and 5.6 kb fragments in Rec1 and Rec2 (Fig. 5.6). These observations were consistent with the insertion in the polyhedrin coding region of a BamHI fragment (2.5 kb) which contains a PstI site. There was no apparent difference between the restriction profiles for Rec1 and Rec2. We conclude that both Rec1 and Rec2 represent the desired recombinants.

5.3.4 Semi-quantitative comparison of viral mRNA levels in *Spodoptera frugiperda* cells infected with recombinant and wild-type baculovirus

Sf9 cells infected with AcNPV produce an abundance of mRNA for the synthesis of polyhedrin protein (1.2 mg/10^6 cells) late in infection (Luckow and Summers, 1988b). The transfer vector used in this study, pVL941 (Fig. 5.1), is not altered in the sequence 5' to the polyhedrin coding region and should generate recombinant AcNPV with a normal polyhedrin promoter activity (Luckow and Summers, 1989). The effect on the level of polyhedrin-MVE mRNA of the insertion of MVE cDNA in the polyhedrin coding region was examined. Levels of polyhedrin mRNA and polyhedrin-MVE mRNA in cells infected with wild-type AcNPV and the recombinant respectively were compared. Cytoplasmic RNA was extracted at 2 days p.i. from cells infected (MOI≈10) with AcNPV, Rec1 or Rec2. ^{32}P-labelled pVL941 was used as a probe for wild-type and polyhedrin-MVE mRNAs; ^{32}P-labelled p2/1/22 was used as probe for polyhedrin-MVE mRNA. ^{32}P-labelled pVL941 hybridized to RNA from cells infected with wild type and recombinant AcNPV (Fig. 5.7). ^{32}P-labelled p2/1/22 only hybridized to RNA from Rec1 and Rec2-infected Sf9 cells (results not shown). The levels of polyhedrin

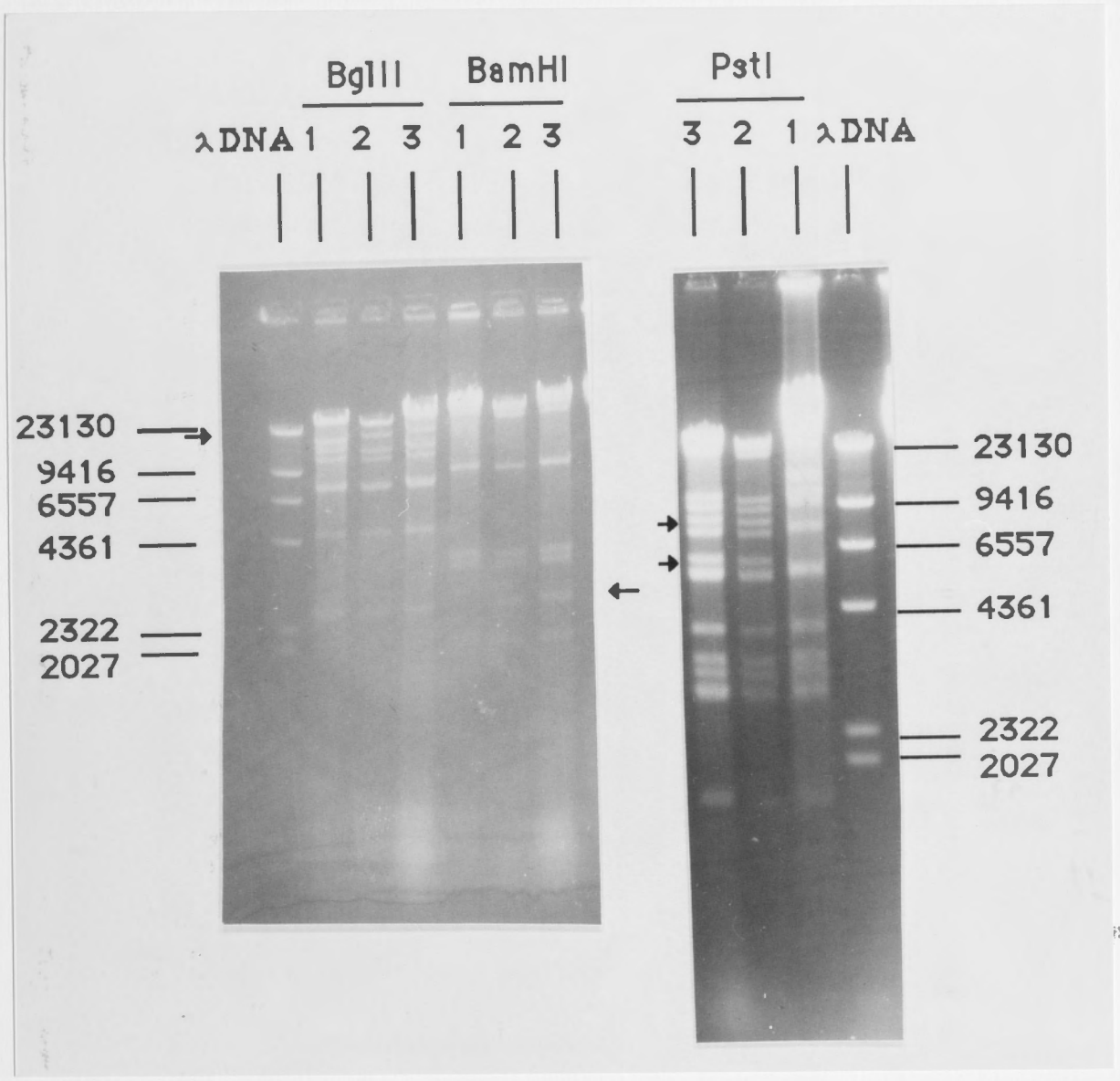


Figure 5.6 Restriction digest profiles of nuclear DNA from Sf9 cells infected with AcNPV (1), Rec1 (2) and Rec2 (3). DNA from 10^5 infected-cells (MOI≈1; 3 days p.i.) was incubated with BamHI, BglII or PstI for 1 hour and electrophoresed on 1% agarose. Detection was by ethidium bromide staining (0.5 μg/ml) and UV illumination. Fragments expected to contain MVE sequences are indicated by arrows.

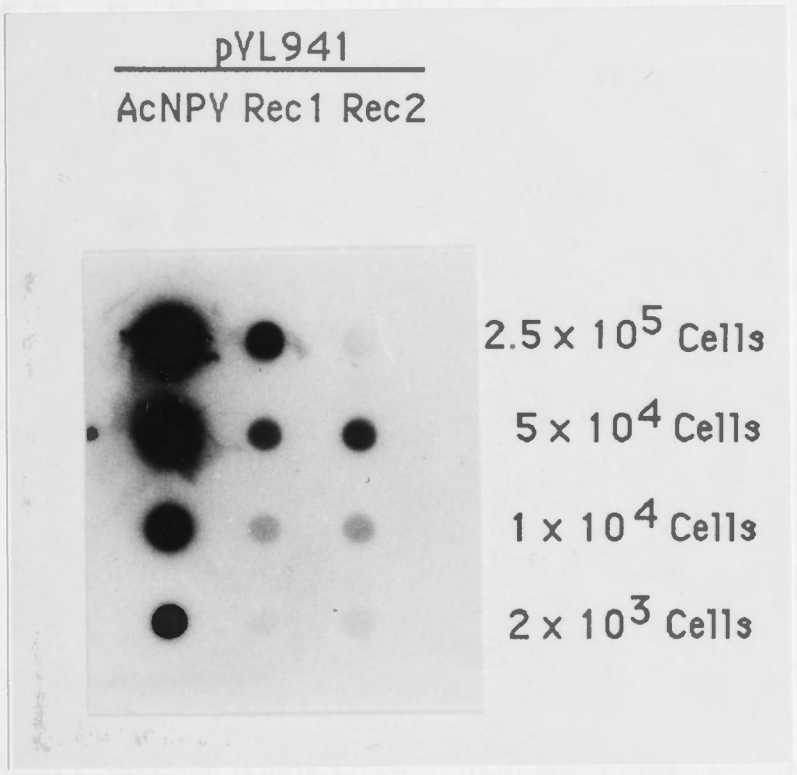


Figure 5.7 Semi-quantitative comparison of mRNA levels in infected Sf9 cells. Cytoplasmic RNA was extracted from Sf9 cells infected with AcNPV, Rec1 or Rec2 (MOI≈10; 48 hours p.i.). Aliquots were dot-blotted on nitrocellulose which was then baked at 80° for 2 hours under vacuum. Detection of polyhedrin mRNA and polyhedrin-MVE mRNA was by hybridization to ^{32}P-labelled pVL941 probe (see text).

mRNA and polyhedrin-MVE mRNA were compared. For hybridization to pVL941, the signals from 2 x 10^3, 10^4 and 5 x 10^4 cells increased with cell number (Fig. 5.7). However the signal from 2.5 x 10^5 Rec2-infected cells was weaker than the signal from fewer cells and this may be due to proteins in the RNA preparation (Luckow and Summers, 1988b). Using the three lower amounts of infected cells as the basis for comparison, similar polyhedrin-MVE mRNA levels were present in Rec1 and Rec2-infected cells (Fig. 5.7). Probing with pVL941, approximately equal intensities were observed for 2 x 10^3 AcNPV-infected cells and 2.5 x 10^5 Rec-infected cells (Fig. 5.7). We conclude that there was approximately 125-fold less polyhedrin-MVE mRNA in Rec-infected cells than polyhedrin mRNA in AcNPV-infected cells.

5.3.5 Expression of MVE structural proteins in *Spodoptera frugiperda* cells infected with recombinant baculovirus

In preliminary studies, the expression of recombinant MVE proteins in Sf9 cells was shown to be maximal between 48 and 120 hours p.i. To characterize the proteins, Sf9 cells infected with Rec1 or Rec2 (MOI≈40) were harvested at 48 hours p.i., lysed and the extract electrophoresed on an SDS-20% PA gel without heat denaturation or reduction. Proteins were transferred to nitrocellulose for detection with anti-MVE HIAF. Lysates of AcNPV-infected Sf9 cells, MVE-infected Vero cells, MVE-infected C6/36 cells and disrupted purified MVE were electrophoresed and blotted as controls. Rec1 and Rec2 gave identical profiles (Fig. 5.8). Four MVE-specific proteins with mol. wts. of approximately 90K, 55K, 40K (trace) and 24K were observed. The most abundant was the 55K protein which corresponded to E in MVE-infected cells and purified virus (Fig. 5.8). The greater mobility of the 55K protein by comparison with E in MVE-infected cells and virus was noted (see below). The 24K protein corresponded to prM in MVE-infected C6/36 cells. PrM in purified MVE migrated as a doublet* and the 24K protein corresponded to the faster migrating band. The 40K protein was detected on immunoblots but was not adequately reproduced on photography; it showed

* This presumably reflects the two forms of prM, one with and one without carbohydrates attached at the single glycosylation site.

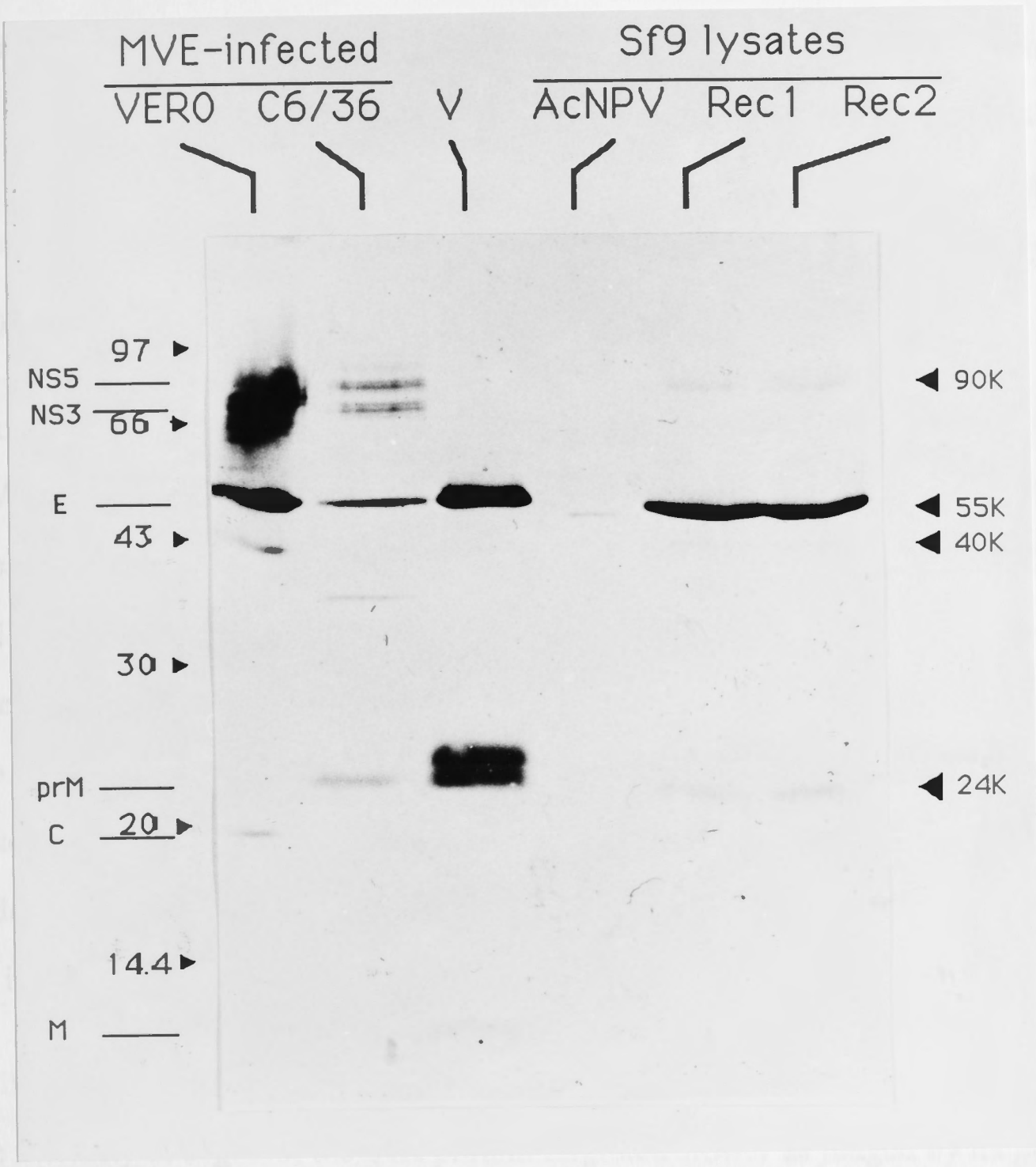


Figure 5.8 Immunoblot analysis of MVE-specific proteins in Sf9 cells infected with wild-type AcNPV, and with Rec1 and Rec2. Infected cells (MOI≈40; 48 hours p.i.) were lysed and aliquots equivalent to 5×10^4 cells separated on an SDS-20% PA gel in the absence of ß-ME and transferred to nitrocellulose. MVE proteins from purified virus (V) containing ≈0.2 µg E, and from MVE-infected Vero and C6/36 cells (MOI≈10) harvested at 48 hours p.i. were controls. Detection was with anti-MVE HIAF. Positions of size markers are are shown. MVE proteins M (8K), C (16K), prM (26K), E (56K), NS3 (70K) and NS5 (103K) are indicated.

variable mobility on SDS-PA gels (results not shown). The 90K protein corresponded in mobility to NS5 but since it derives from cDNA without this region of the genome, it cannot be NS5. The identities of the 90K and 40K proteins are discussed further below. MVE-specific proteins were not detected in culture medium equivalent to 5×10^4 cells, indicating that the proteins were mainly cell-associated (results not shown).

To determine the time course of protein synthesis, cells were infected with AcNPV, Rec1 or Rec2 (MOI≈40) and incubated for 18, 24, 48, 72, 96 or 120 hours. Cell lysates were electrophoresed without prior heating or reduction on an SDS-20% PA gel and the proteins transferred to nitrocellulose for detection by HIAF. No proteins were detected in AcNPV-infected cells. MVE-specific proteins were detected at 18 hours p.i. The relative intensities of the four MVE-specific proteins did not vary with time after infection. The profiles for Rec1 and Rec2-infected cells at 18-120 hours p.i. were identical. Proteins of 55K, 40K and 24K were observed (results for Rec1 are shown in Fig. 5.9).

Differences are seen between Figs. 5.8 and 5.9. The 40K protein is of stronger relative intensity in Fig. 5.8; the 24K protein migrated slower as judged by its position relative to the prM doublet in purified MVE; and the 90K protein was not detected in Fig. 5.9. Both experiments used unreduced proteins denatured in 0.1% SDS; immunoblots were performed under similar conditions. We believe that the different mobilities and reactivities with HIAF resulted from differences in the extent of denaturation during electrophoresis or of renaturation during immunoblotting.

5.3.6 Proteolytic processing and glycosylation of recombinant MVE proteins in *Spodoptera frugiperda* cells

To define the pathway of proteolytic cleavage in Sf9 cells and to assess its efficiency, infected cells were pulse-labelled and lysates examined after various lengths

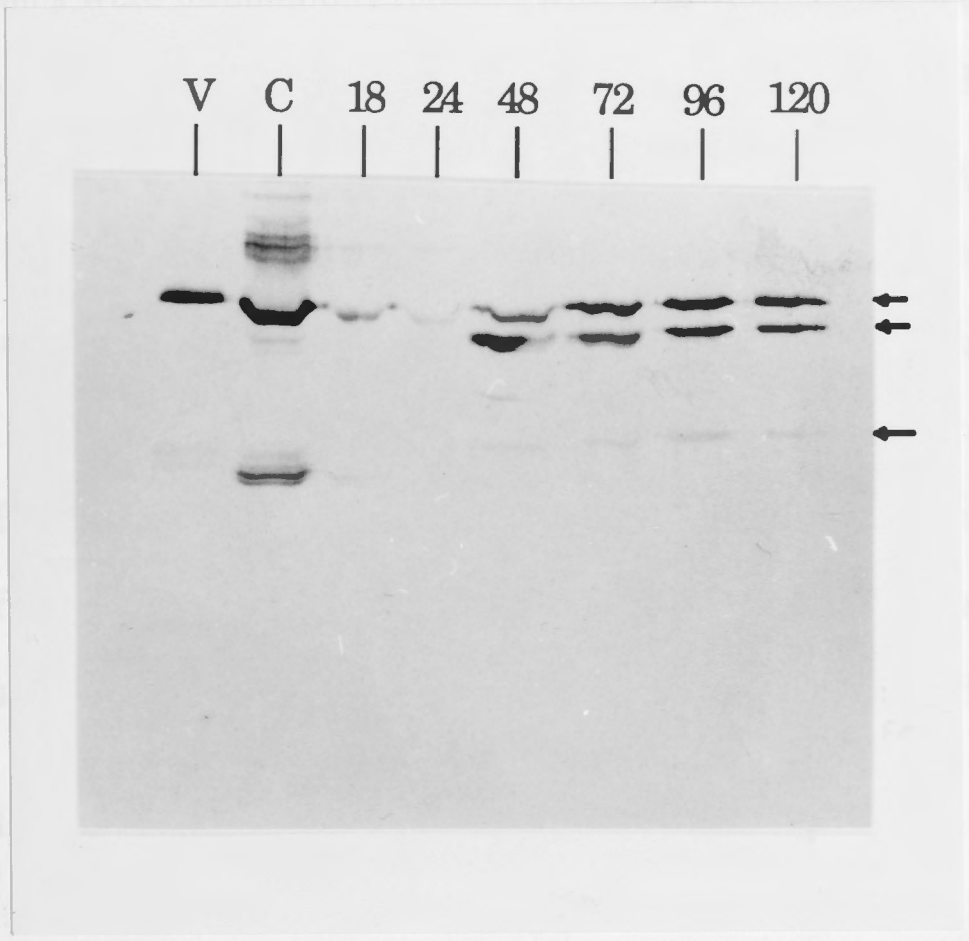


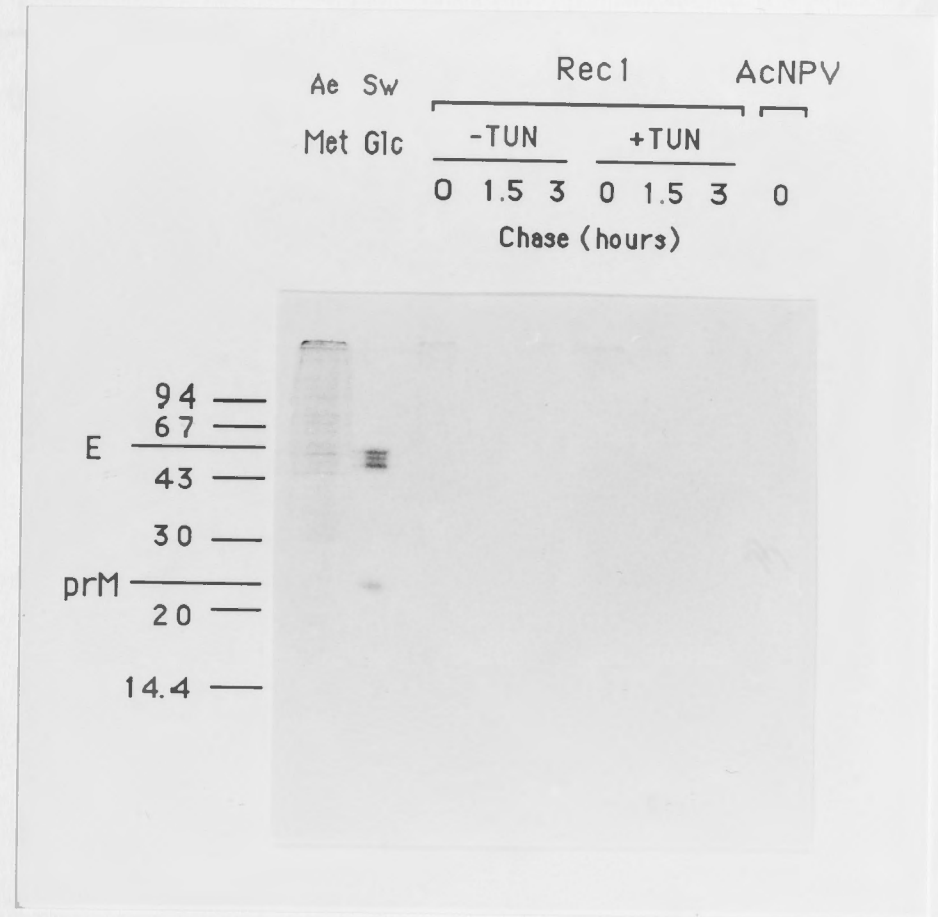
Figure 5.9 Time course of MVE-specific protein synthesis in Rec1-infected Sf9 cells. Cells were infected with AcNPV or Rec1 in 35 mm Petri dishes (MOI≈40) and incubated for 18, 24, 48, 72, 96 and 120 hours at 28°. Cells were lysed and aliquots of total lysate corresponding to 5×10^4 cells were electrophoresed on an SDS-20% PA gel under non-reducing conditions for immunoblot analysis using anti-MVE HIAF (as in Fig. 5.8). C, MVE-infected (MOI≈10) Vero cell lysate (48 hours p.i.; 10^5 cells); V, purified MVE containing ≈0.1 μg E. Arrows to the left of the photograph indicate the 55K, 40K and 24K proteins in Sf9 cell lysates.

of chase. Preliminary studies showed that the synthesis of both polyhedrin and MVE proteins was most rapid from 24-48 hours p.i.; synthesis of cellular proteins was inhibited at 48 hours p.i. (results not shown). In further studies, Sf9 cells were infected with Rec1 or AcNPV (MOI≈40) and labelled with ^{35}S-methionine for 10 min at 48 hours p.i. followed by a chase of 1.5 or 3 hours with unlabelled methionine and cycloheximide. Lysates were immunoprecipitated with anti-MVE HIAF and the proteins separated by SDS-PAGE (Fig. 5.10). Control lysates were MVE-infected SW13 (human adenocarcinoma) cells labelled (from 24-36 hours p.i.) with ^{3}H-glucosamine and MVE-infected C6/36 cells labelled (21-24 hours p.i.) with ^{35}S-methionine. The four MVE glycoproteins labelled in SW13 cells were E (56K), NS1 (50K and 46K) and prM (26K) (Fig. 5.10A; see also Lee *et al.*, 1990). E generated in C6/36 cells migrated faster (54.5K) than E generated in SW13 cells. The only protein immunoprecipitated in AcNPV-infected Sf9 cells was a 35K protein (Fig. 5.10B), probably the polyhedrin protein. The immunoprecipitation of this protein appeared to be non-specific.

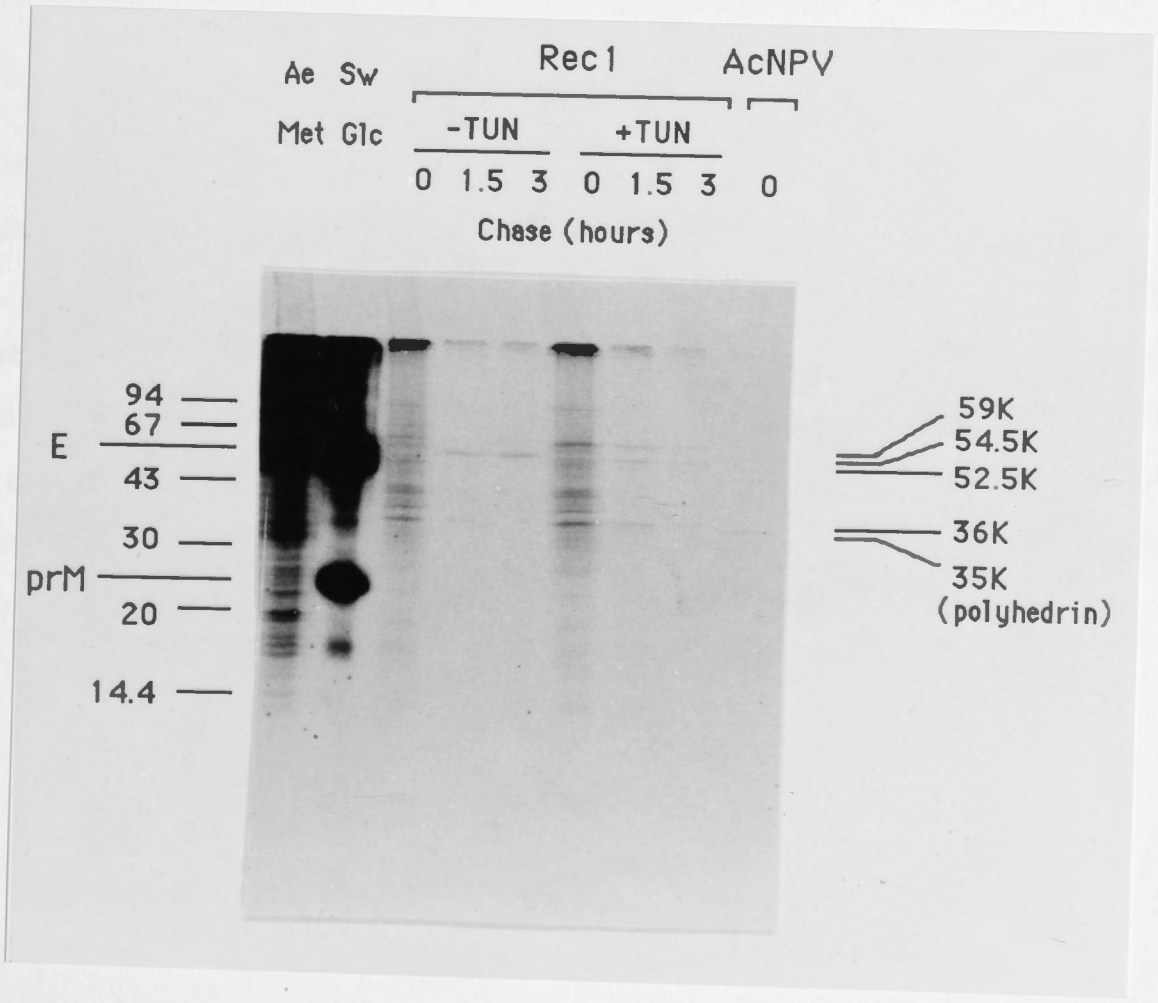
In cells infected with Rec1, a complex pattern was observed in pulse-chase experiments (Fig. 5.10). After a 10 min labelling period, a number of relatively large proteins of nominal mol. wts. 90, 60 and 58K were observed. Other proteins of lower mol wts. (36-50K) were seen. Following a chase, the 90, 60 and 58K proteins disappeared as did all the small proteins except the 36K protein. Only two proteins were detected after a 1.5 hour chase: a protein of 54.5K and the 36K protein. Only the 54K protein was stable after a 3 hour chase. The 54.5K protein is E on the basis of its size, absence in tunicamycin-treated cells and reactivity with mAbs (see below). Proteins corresponding to prM, M and C were not detected in infected cells after a pulse-chase. The identification of the other labelled proteins is less certain. Fig. 5.11 shows a possible processing pathway for the C-prM-E-NS1-polyhedrin polyprotein. The 90K protein may represent the full length polyprotein with or without the NS1-polyhedrin

Figure 5.10 Processing of MVE proteins in Sf9 cells. Cells in 24-well trays (5 x 10^5 cells/well) were infected with Rec1 or with AcNPV (MOI≈40) and incubated at 28°. At 48 hours p.i., cells were labelled with ^{35}S-methionine (80 µCi/ml) for 10 min without or with tunicamycin (10 µg/ml). Medium containing an excess of unlabelled methionine and cycloheximide (0.1 mg/ml final concentration) was then added and incubation continued for 1.5 or 3 hours. Control lysates were from MVE-infected SW13 cells labelled (12 hours) with ^{3}H-glucosamine and MVE-infected C6/36 cells labelled (2 hours) with ^{35}S-methionine. Lysates were immunoprecipitated with anti-MVE HIAF for analysis by SDS-PAGE and fluorography. Positions of size markers are shown. E and prM proteins in C6/36 cells and in SW13 cells are indicated. Ae, *Aedes albopictus* (C6/36) cells; Met, ^{35}S-methionine labelled; Sw, SW13 cells; Glc, ^{3}H-glucosamine labelled. (A) and (B) are autoradiographs of the same gel exposed for 1 and 14 days respectively.

A.


Ae Sw
Rec1
AcNPV
Met Glc
-TUN
+TUN
0 1.5 3 0 1.5 3 0
Chase (hours)
94
67
E
43
30
prM
20
14.4

B.


Ae Sw
Rec1
AcNPV
Met Glc
-TUN
+TUN
0 1.5 3 0 1.5 3 0
Chase (hours)
94
67
E
43
30
prM
20
14.4
59K
54.5K
52.5K
36K
35K
(polyhedrin)

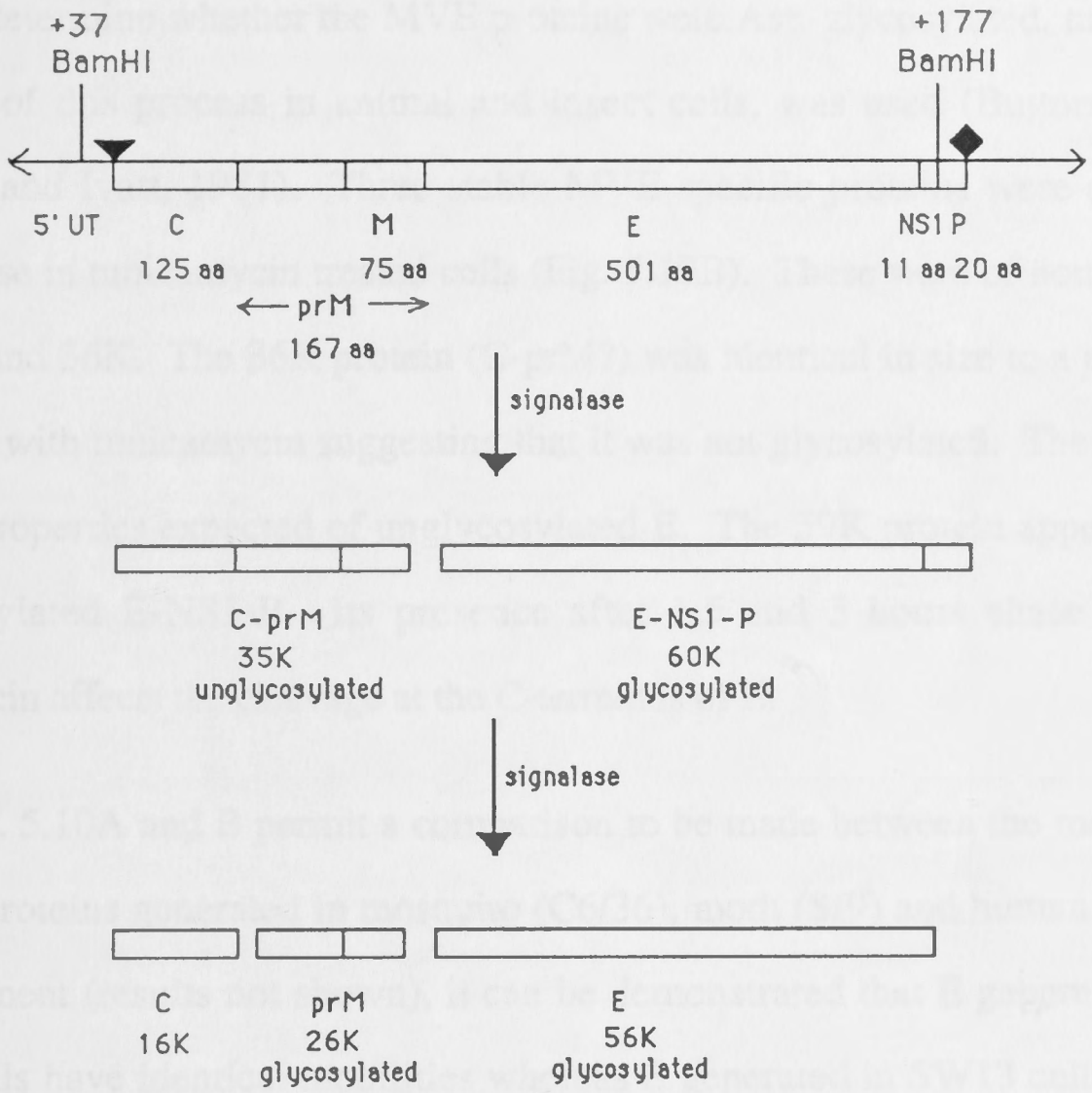


Figure 5.11 MVE coding region in recombinant AcNPV showing possible proteolytic processing pathway. The MVE cDNA inserted in the polyhedrin coding region of AcNPV is shown. It contains the 5' untranslated region of MVE genome (≈90 nucleotides), and coding regions for C (375 nucleotides), prM (501 nucleotides), E (1503 nucleotides) and part of NS1 (34 nucleotides). The predicted size (in amino acids) and mol. wts. of prM and E glycoproteins and of C are indicated. Sequence and protein data are from Dalgarno *et al.* (1986) and Lee *et al.* (1990). The ORF begins with the initiation codon (triangle) and ends with TAA (diamond). The position of the termination codon was deduced on the basis of sequence data for pVL941 and for the polyhedrin coding region (Summers and Smith, 1987).

fragment at the C-terminus. The 58-60K proteins may be the E-NS1-polyhedrin and the 36K protein C-prM.

To determine whether the MVE proteins were Asn-glycosylated, tunicamycin, an inhibitor of this process in animal and insect cells, was used (Butters *et al.*, 1981; Hubbard and Ivatt, 1981). Three stable MVE-specific proteins were observed after pulse-chase in tunicamycin treated cells (Fig. 5.10B). These were of nominal mol. wts. 59, 52.5 and 36K. The 36K protein (C-prM?) was identical in size to a protein in cells untreated with tunicamycin suggesting that it was not glycosylated. The 52.5K protein had the properties expected of unglycosylated E. The 59K protein appeared to be the unglycosylated E-NS1-P. Its presence after 1.5 and 3 hours chase suggests that tunicamycin affects the cleavage at the C-terminus of E.

Figs. 5.10A and B permit a comparison to be made between the mobilities of the MVE E proteins generated in mosquito (C6/36), moth (Sf9) and human (SW13) cells. By alignment (results not shown), it can be demonstrated that E generated in Sf9 and C6/36 cells have identical mobilities whereas E generated in SW13 cells is of slightly lower mobility.

We have noted differences in the profiles of MVE proteins in Fig. 5.8 and Fig. 5.10B. The detection of proteins was by the same HIAF in both cases. However in Fig. 5.8, unreduced proteins in lysates were electrophoresed, transferred to nitrocellulose and reacted with antibodies. In Fig. 5.10B, labelled native proteins in lysates were reacted with antibodies and then reduced and electrophoresed. The 54.5/55K protein and the 90K protein was observed in both, but the 24K and 40K proteins in Fig. 5.8 were not in Fig. 5.10B. The 24K protein may not be detected by RIP in Fig. 10B due to less effective labelling by ^{35}S-methionine relative to the larger proteins. We do not consider the size estimation of the 40K protein to be accurate as it was based on mobilities of unreduced proteins in SDS-PAGE and the 40K protein

showed variable mobilities in such analysis (see above). It is likely that this protein corresponded to the 36K protein in Fig. 5.10B (see above).

5.3.7 Reactivity of E protein with neutralizing mAbs

The 54.5K protein corresponded in size to the MVE E protein and it was also Asn-glycosylated. It was also of interest to examine the reactivity of this protein with neutralizing mAbs to confirm that it was E and as an index of conformation. MAbs against five different epitopes (E-1c, E-1d, E-5b, E-7 and E-8) were available and these reacted strongly with the unreduced authentic MVE E protein (Chapter 3). Lysates of Sf9 cells infected with Rec1 and with AcNPV (MOI≈10; 48 hours p.i.) were electrophoresed, unreduced, on an SDS-20% PA gel. Control lysates (unreduced) of MVE-infected C6/36 and Vero cells were electrophoresed on the same gel. Proteins were transferred to nitrocellulose for detection by mAbs. Unreduced recombinant E reacted with all five mAbs: no protein in AcNPV-infected Sf9 cells reacted with any mAb (Fig. 5.12). Reactivity was strongest with anti-E-5b and E-8, and moderate to weak with anti-E-7, E-1d and E-1c. The same pattern of reactivity was obtained with E in MVE-infected C6/36 and Vero cells (Fig. 5.12). These results suggested that recombinant E was similar in conformation to authentic E.

5.3.8 Localization of E protein in *Spodoptera frugiperda* cells

The MVE-specific proteins which reacted with anti-MVE HIAF were not secreted in quantity from Sf9 cells. To explore the subcellular localization of the MVE proteins, nuclei from infected Sf9 cells were first examined. Rec1-infected cells were lysed with 1% NP-40. Lysates were centrifuged and nuclei pelleted and resuspended in RIP buffer. Nuclear extracts were dot-blotted on nitrocellulose and reacted with anti-MVE HIAF. No MVE-specific proteins were detected in a nuclear extract from 5×10^5 Sf9 cells even though total lysates from a similar number of cells contained MVE proteins (results not shown).

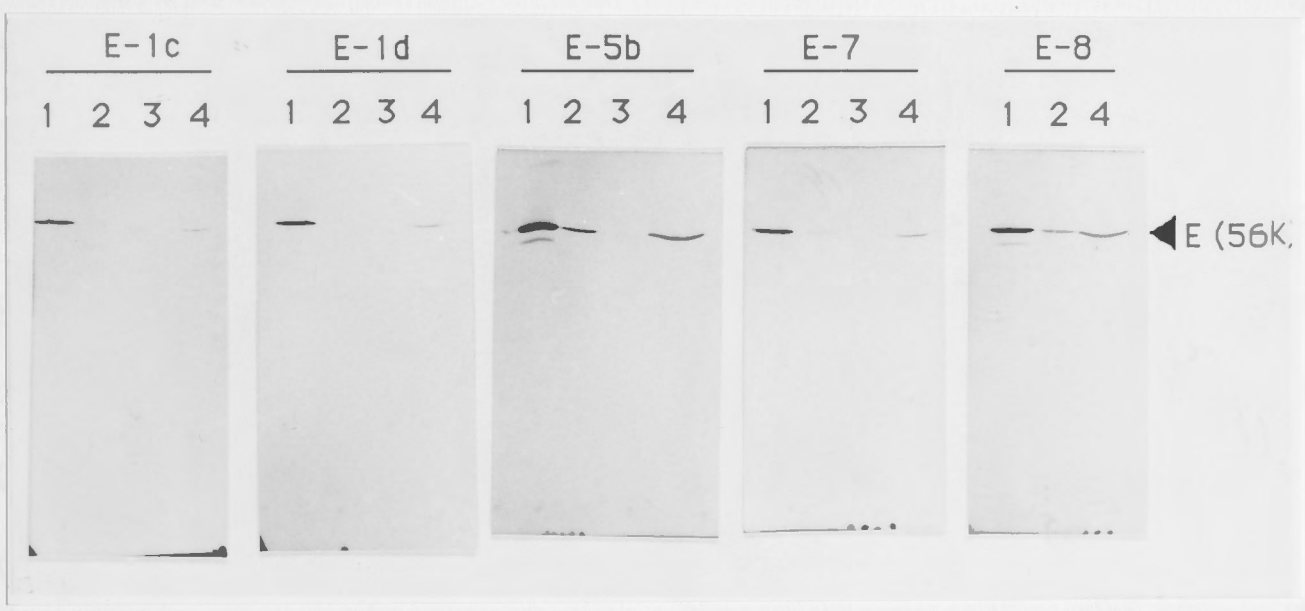


Figure 5.12 Immunoblot analysis of E protein synthesized in Sf9 cells using anti-E mAbs. Lysates of cells infected with Rec1 and AcNPV were prepared as in Fig. 5.8, electrophoresed on an SDS-20% PA gel in the absence of ß-ME and transferred to nitrocellulose which was reacted with anti-E-1c, E-1d, E-5b, E-7 and E-8 mAbs (see Table 3.1). Lane 1, MVE-infected Vero cells (2×10^5); lane 2, MVE-infected C6/36 cells (5×10^4); lane 3, AcNPV-infected Sf9 cells (5×10^4); lane 4, Rec1-infected Sf9 cells (5×10^4).

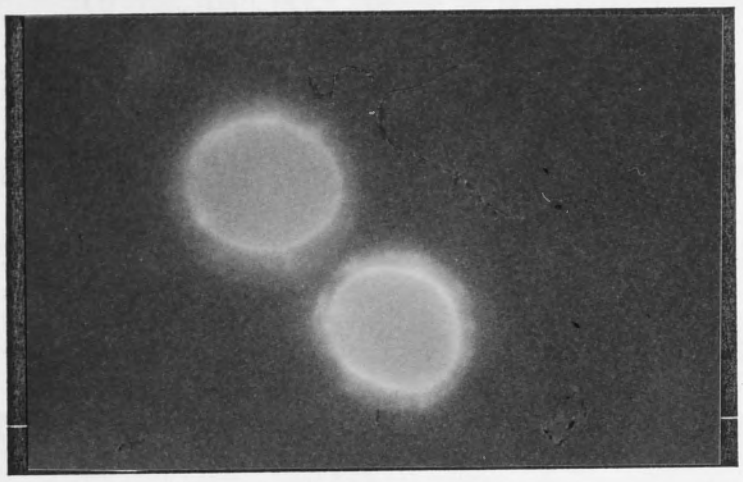
Immunofluorescence was used to localize the E protein in the extranuclear region. Two approaches for fixation were examined. The first (paraformaldehyde fixation) was used to localize internal antigens (Harlow and Lane, 1988); the second (paraformaldehyde plus glutaraldehyde) was used to detect cell surface antigens (Hardham, 1985).

Paraformaldehyde method: Sf9 cells on coverslips were infected with AcNPV or with Rec1 (MOI≈10). Control C6/36 cells were infected with MVE (MOI≈10). At 48 hours p.i., coverslips were fixed in 4% paraformaldehyde, permeabilized with Triton X and reacted with anti-E-5b mAb. FITC-conjugated anti-(mouse IgG) was added and immunofluorescence detected by UV microscopy. Fluorescence was observed in the cytoplasm of Rec1- but not AcNPV-infected Sf9 cells (Fig. 5.13A, B). The intensity was strongest in the perinuclear region. The MVE-infected C6/36 cells were more weakly stained than the Rec1-infected Sf9 cells, but a similar distribution of E was seen (Fig. 5.13C). The nuclei in infected C6/36 cells were not enlarged as in infected Sf9 cells. No fluorescence was seen in mock-infected C6/36 cells (Fig. 5.13D).

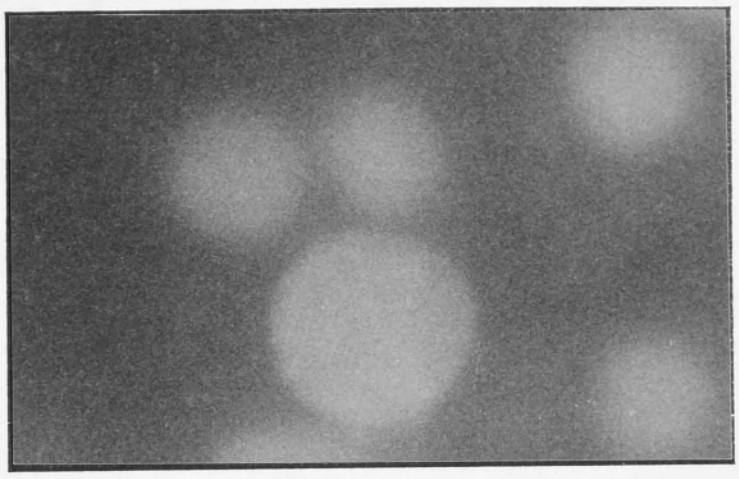
Paraformaldehyde-glutaraldehyde fixation: Coverslips infected as above were fixed in paraformaldehyde (4%)-glutaraldehyde (0.2%), and reacted with anti-E-5b mAb and FITC-conjugated anti-(mouse IgG). Approximately half the Rec1-infected Sf9 cells were stained at the cell membrane (Fig. 5.13E, F), indicating that the recombinant E was located on the surface of infected cells. Some infected cells were permeabilized by this technique and perinuclear fluorescence was detected in these cells (Fig. 5.13E). MVE-infected C6/36 cells showed staining predominantly in the cytoplasm due to permeabilization (Fig. 5.13G, H). The staining was weak and it could not be ascertained whether E was absent from the surface. From both types of fixation technique, we conclude that there was less intense staining overall in C6/36 than in Sf9 cells. This was probably due to lower amounts of E being produced in C6/36 cells.

Figure 5.13 Immunofluorescence localization of MVE E protein in infected cells. Sf9 cells on glass coverslips were infected with Rec1 (MOI≈10; A, E) or with AcNPV (MOI≈10; B, F). C6/36 cells were infected with MVE (MOI≈10; C, G) or mock infected (D, H). At 48 hours p.i., coverslips were fixed in paraformaldehyde (A, B, C, D) for detection of internal antigens or paraformaldehyde-glutaraldehyde (E, F, G, H) for detection of cell surface antigens. The coverslips were reacted with anti-E-5b mAb. FITC-anti-(mouse IgG) was used for immunofluorescence detection. The photographs were taken at magnifications of 1000X (E, F) or 400X (A, B, C, D, G, H) under microscopy.

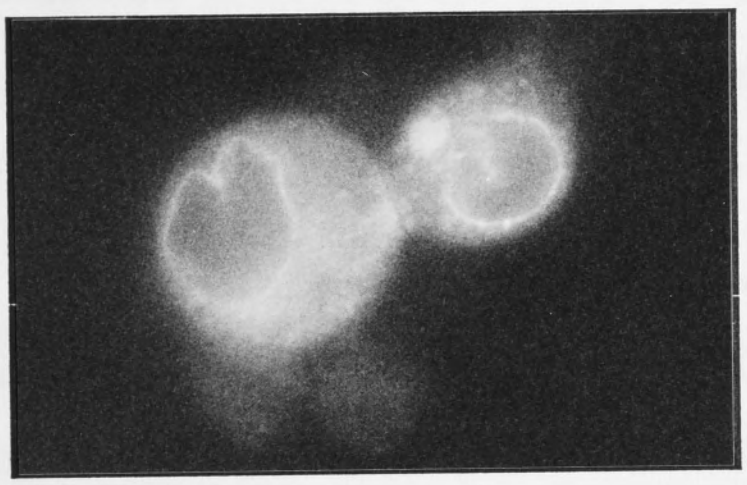
A.



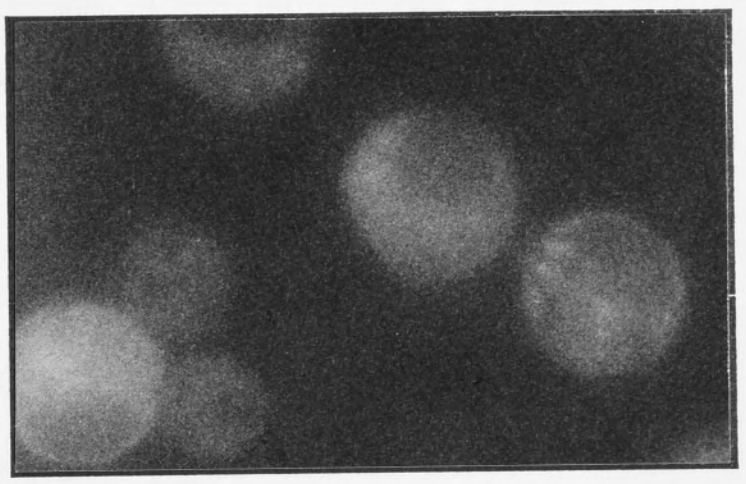
B.



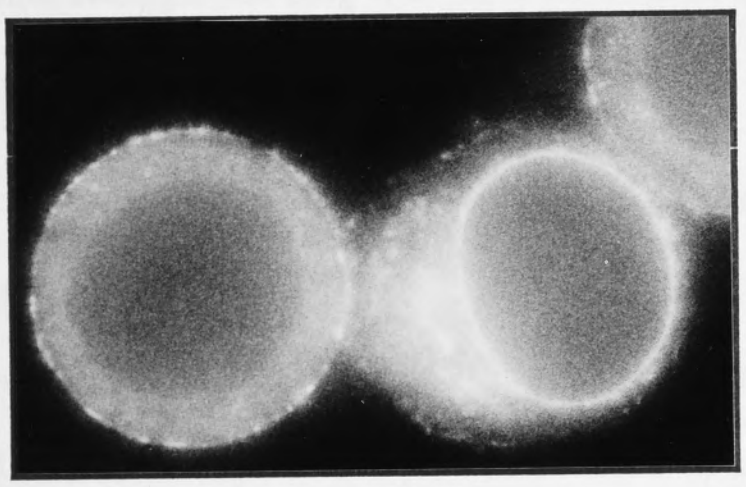
C.



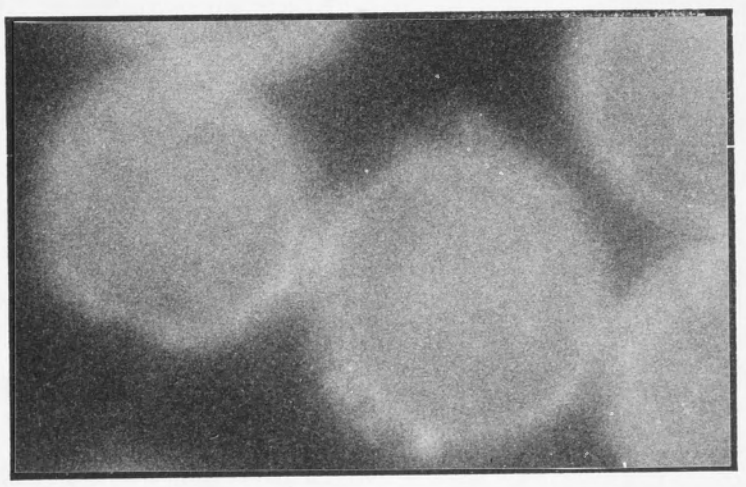
D.



E.

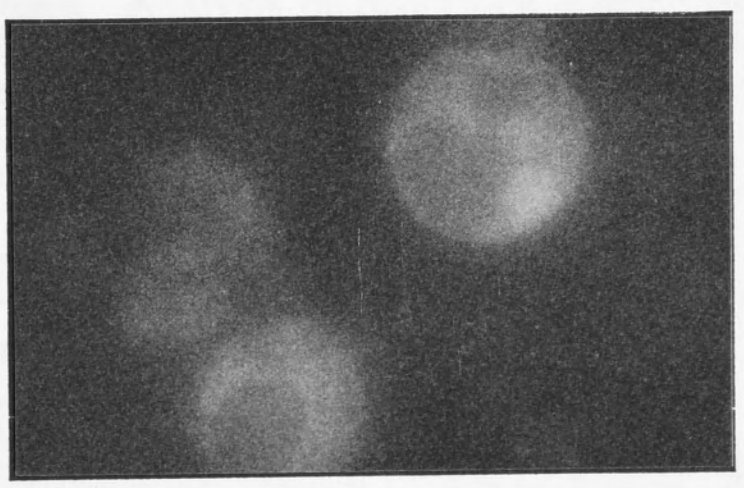


F.



G

H.



5.3.9 Yield of E protein from *Spodoptera frugiperda* cells

An attempt was made to quantitate levels of recombinant E generated in Sf9 cells. The method was based on a comparison of relative reactivities of HIAF and anti-E-5b mAb with standardized amounts of purified MVE and with infected Sf9 cell lysates. MVE was purified by gradient centrifugation (Chapter 3). Analysis by SDS-PAGE showed that the preparation was approximately 50% viral protein and approximately 50% bovine serum albumin. Quantitation of the MVE proteins was by Bio-Rad colorimetric assay (Chapter 3). The results of a number of immunoblotting experiments with anti-MVE HIAF and anti-E-5b mAb indicated that the yield of E was ≈4 $\mu g/10^6$ cells (data not shown).

In related experiments using the same procedures, the yield of E from MVE-infected C6/36 cell lysates was approximately one third of that in Sf9 cells. This is consistent with the results of immunofluorescence staining of infected C6/36 and Sf9 cells; stronger staining was demonstrated in the Sf9 cells.

5.3.10 Summary

A recombinant baculovirus, the *Autographa californica* nuclear polyhedrosis virus, containing the MVE structural protein genes downstream from the polyhedrin promoter was generated by homologous recombination between the recombinant vector pVL941-MVE and baculovirus DNA. A selection procedure based on detection of MVE cDNA allowed the isolation of three recombinant viruses. Analysis of restriction digest profiles, mRNA synthesis and MVE proteins for two of these demonstrated that they were identical. Infection of Sf9 cells with recombinant virus produced polyhedrin-MVE mRNA at a level which was approximately 125-fold less than that of polyhedrin mRNA in AcNPV-infected Sf9 cells. Several proteins were detected by anti-MVE HIAF in Sf9 cells infected with recombinant baculovirus. A ≈55K protein corresponded in size to E and reacted with neutralizing mAbs against MVE E protein. We have tentatively identified this protein as E; however sequence at the N- and C-termini is needed to verify

that correct cleavage has occurred; the use of the term 'E' must therefore be considered provisional. A 24K protein observed in immunoblots may be prM but was not seen in labelling experiments. Other proteins of 40K and 90K were probably high mol. wt. precursors. MVE proteins were detected from 18-120 hours p.i. Proteolytic processing was studied in pulse-chase experiments; these demonstrated efficient cleavage of polyprotein precursors into a 54.5K protein. Use of tunicamycin demonstrated that the 54.5K protein underwent Asn-linked glycosylation consistent with the properties of E. Cleavage of E-NS1-polyhedrin was incomplete in the presence of tunicamycin. The five neutralizing mAbs had reactivity patterns against the 54.5K protein similar to those against authentic E in MVE-infected cells. Localization by immunofluorescence staining with anti-E-5b mAb showed that E was distributed in Sf9 cells in the perinuclear region and at the cell surface. The yield of recombinant E was approximately 4-5 μg per 10^6 cells; this was estimated by its reactivity with HIAF relative to a standardized amount of purified MVE. On the basis of reactivities with HIAF and immunofluorescence staining with anti-E-5b mAb, we conclude that the yield of E from Sf9 cells was significantly greater per cell than from MVE-infected C6/36 cells.

5.4 DISCUSSION

The aim of this study was to explore the expression of cDNA encoding the MVE structural proteins in the baculovirus expression system. Of major interest was the possibility of generating E protein in a 'native' conformation and in good yield. The method used involved the introduction into the transfer vector pVL941, of MVE cDNA encoding the 5' untranslated region, C, prM, the complete E gene and a small portion of NS1. Cotransfection of a lepidopteran cell line (Sf9) with recombinant plasmid and with baculovirus DNA generated the desired recombinant baculovirus, which was isolated using a strategy based on hybridization of infected cell DNA with labelled MVE cDNA. This method was superior to two other methods tested and led to the isolation of

two identical recombinants. We estimate that the yield of the AcNPV-MVE recombinant was of the order of 0.1% of the total cotransfection progeny.

Processing of recombinant MVE proteins in *Spodoptera frugiperda* cells

Proteins corresponding in size to prM and E were detected in Sf9 cells infected with the recombinant AcNPV. The identity of recombinant E was confirmed using anti-E mAbs (see below). The other MVE-specific proteins seen in Sf9 cells were probably high mol. wt. precursors and processing intermediates. These were tentatively identified by their mol. wts. as the following polyproteins: C-prM-E-NS1-poly, E-NS1-poly and C-prM (the terms 'NS1' and 'poly' denote 11 and 20 amino acids of the NS1 and polyhedrin proteins respectively; see below).

The processing events in flavivirus-infected cells which produce the structural proteins include two types of cleavage; C, prM and E are produced from the polyprotein by signalase cleavage in the lumen of the RER, where E and prM also undergo Asn-linked glycosylation (Chapter 1). The expression of MVE E and prM in Sf9 cells infected with recombinant baculovirus is consistent with cleavage by signalase. We conclude that the polyprotein encoded in the recombinant baculovirus underwent translocation into the lumen of the RER, probably mediated by the signal sequences at the C-termini of C, prM and E, where cleavage by signalase produced the prM and E proteins. Although C was not detected in infected Sf9 cells, the appearance of prM and E proteins indicated that C was probably cleaved from the polyprotein precursor. The failure to detect C suggests that it was degraded after cleavage from the polyprotein. In a study of processing of Sindbis structural proteins in Sf9 cells using the AcNPV expression system (Oker-Blom and Summers, 1989), the capsid (C) protein is partly degraded. Since the C proteins of both Sindbis and MVE are thought to bind their respective viral genome RNAs soon after synthesis, C may be susceptible to degradation in Sf9 cells in the absence of genome RNA.

The second type of cleavage for the structural proteins converts prM to M (Chambers *et al.*, 1990a). This occurs late in the virus maturation pathway or during virus release; the cleavage is frequently incomplete as released flavivirus particles commonly contain prM as well as M (Chater 1). We did not detect free M in Sf9 cells. This may relate to the fact that events in virus maturation which are important for this cleavage are not occurring in our system. However M is often not detected in cells infected with MVE and other flaviviruses (Lee *et al.*, 1990; Shapiro *et al.*, 1972; Westaway, 1987). Another explanation for our failure to detect M relates to the methods of labelling with ^{35}S-methionine and of immunoblotting used for detection: MVE M may not be strongly labelled as it contains only one methionine residue (Dalgarno *et al.*, 1986); losses of M during transfer or blotting may occur due to its small mol. wt. (8K).

We have assessed the kinetics of processing of MVE proteins in Sf9 cells by pulse-chase experiments. Cleavage to generate the 54.5K protein occurred efficiently in Sf9 cells and was complete well within 1.5 hours. Signalase cleavage at the N-terminus of prM was incomplete as the putative C-prM intermediate was detected after chase. The Asn-linked glycosylation of MVE proteins in Sf9 cells was examined using tunicamycin. The occurrence of Asn-linked glycosylation of E indicated that it was translocated into the lumen of the RER. We could not detect prM by labelling and thus its glycosylation could not be confirmed. C-prM was detected by pulse-labelling, but it appeared to be unglycosylated; an explanation for its lack of glycosylation and cleavage is that it was not translocated into the lumen of the RER. The inefficiency in the cleavage of E-NS1-poly into E in tunicamycin-treated cells suggested that, in the absence of the attachment of glycans in the lumen, signalase cleavage at the C-terminus of E occurs inefficiently. Tunicamycin did not affect signalase cleavage at the N-termini of prM and E. Inhibition of glycosylation has been demonstrated to impair intracellular protein transport and cause aberrant protein folding (Leavitt *et al.*, 1977; Machamer and

Rose, 1988). It is therefore possible that the conformation of unglycosylated E may have affected signalase activity.

The observation of different mobilities of E generated in insect and primate cells may be related to a difference in composition of glycans in E. The MVE E protein (54.5K) in Sf9 cells has a similar mobility to E in MVE-infected C6/36 cells. E in MVE-infected Vero and SW13 cells appeared to be slightly larger. Insect cells such as C6/36 and Sf9 produce high mannose glycoproteins which are deficient in complex carbohydrates and may thus be smaller by comparison with glycoproteins in mammalian cells such as SW13 and Vero (Hsieh and Robbins, 1984; Kornfeld and Kornfeld, 1985).

Conformation of recombinant E protein

The properties of the recombinant E from Sf9 cells were compared with those of authentic E to assess the usefulness of the baculovirus system in producing properly folded, functional MVE proteins. Antigenically, the recombinant protein could not be differentiated from authentic E in MVE-infected cells on the basis of its reactivity with five neutralizing mAbs which defined MVE epitopes E-1c, E-1d, E-5b, E-7 and E-8. E-1c, E-1d, E-5b and E-8 are probably all in domain A (Chapter 4). The anti-E-7 mAb defines a spatially distinct epitope, perhaps in domain B, but it has not been mapped. Epitopes in domain A are more sensitive to conformational changes induced by SDS, pH and disulfide reduction than are epitopes in domains B and C. Our data therefore suggest that this conformationally labile domain in E is reproduced in the recombinant 54.5K protein.

In a study of JE structural protein synthesis in Sf9 cells using a baculovirus vector (Matsuura *et al.*, 1989), two constructs, expressing prM (minus the N-terminus)-E-NS1 (N-terminus only) or C (minus the N-terminus)-prM-E-NS1 (N-terminus only) were compared for reactivities with nine E-specific mAbs (five were neutralizing). The

construct which expressed undeleted prM and E reacted with all nine mAbs by immunofluorescence; reactivities were similar to those seen with JE-infected cell lysates. The construct expressing deleted prM and intact E reacted with only eight mAbs, three of which showed decreased reactivities. As the E protein expressed for both constructs is glycosylated and was generated in similar quantity, the different mAb reactivities suggest a possible role for prM in the correct folding of JE E protein.

Yield of recombinant E

In our studies we have estimated that the yield of E protein in recombinant infected Sf9 cells was around 4-5 $\mu g/10^6$ cells, as judged using partially purified MVE as a standard. Comparisons were made between similar numbers of recombinant-infected Sf9 cells and MVE-infected C6/36 cells in terms of immunoreactivity with HIAF against MVE. We found that the yield of E in C6/36 cells is approximately one third of that in Sf9 cells. Other expression studies on JE and DEN-4 proteins in Sf9 cells using baculovirus vectors (Matssura *et al.*, 1989; Zhang *et al.*, 1988) reported that yields of recombinant proteins were similar or lower than those in virus-infected cells. The available data on the expression of MVE, JE and DEN-4 envelope proteins in Sf9 cells therefore suggest that yields are approximately 500-fold lower than yields of polyhedrin protein in wild-type infected cells (1.2 $mg/10^6$ cells; Summers and Smith, 1987). Although it would be possible to propagate large quantities of cells in suspension cultures using new techniques of culturing Sf9 cells (see review by Cameron *et al.*, 1989), it is also possible that by altering the recombinant construct increased yields may be readily obtained.

We have therefore explored some of the possible explanations for the low yield. It was probably not a result of protein degradation as the level of E was constant late in infection when little synthesis of MVE proteins occurred. The processing of precursors into E also appeared to be complete (see above). Given that the level of polyhedrin-MVE mRNA in infected Sf9 cells was about 125-fold less than the level of polyhedrin

mRNA in AcNPV-infected Sf9 cells, the low level of polyhedrin-MVE mRNA was probably the dominant factor in determining the yield. pVL941 was not expected to be responsible for a low level of recombinant polyhedrin mRNA during infection. This vector has been used to express *E.coli* chloramphenicol acetyltransferase and ß-galactosidase, and the level of the corresponding mRNA is similar to the level of polyhedrin mRNA in wild type AcNPV-infected cells (Luckow and Summers, 1989). The low mRNA level for MVE may be related to the activity of the polyhedrin promoter, processing of transcript or the rate of degradation of mRNA. The introduction of MVE cDNA results in the insertion of an extra 90 nucleotides of untranslated sequence between the polyhedrin promoter and the region to be translated into MVE polyprotein. It also creates a much larger polyhedrin mRNA (2.5 kb) than the original construct (≈0.7 kb). These two factors may have affected polyhedrin promoter activity, post-transcriptional processing or the stability of hybrid mRNA.

For the expression of the DEN-4 and JE proteins, the transfer vectors pAc373 and pAcYM1 respectively were used (Matsuura et al., 1989; Zhang et al., 1988). The former is deleted in the sequence immediately upstream of the polyhedrin initiation codon and results in a reduced promoter activity compared to the wild-type polyhedrin promoter (Luckow and Summers, 1988b); pAcYM1 has been used to generate high yields of foreign proteins in Sf9 cells (Matsuura *et al.*, 1987). The recombinant baculovirus constructs used for MVE, DEN-4 and JE also differed in the lengths of viral noncoding and coding sequences downstream of the polyhedrin promoter. As the level of mRNA was not reported for the DEN-4 and JE studies, it is not clear if low mRNA level has caused poor expression.

Results from our study suggest that in order to improve expression of MVE recombinant E, the level of polyhedrin-MVE mRNA has to be raised. To do this recombinant AcNPV which contains different sequences flanking the E gene could be constructed and examined for expression.

Localization of recombinant E in Sf9 cells

From our study it is clear that the MVE E protein accumulates in the perinuclear region of infected cells as well as at the cell surface. This perinuclear accumulation was essentially the same as that seen in MVE-infected C6/36 cells. Perinuclear staining has also been observed in KUN- and DEN-2-infected Vero cells (Westaway and Goodman, 1987), in YF-infected Vero cells (Gould *et al.*, 1985) and in DEN-2 infected LLC-MK2 cells (Cardiff *et al.*, 1973). The appearance of E at the surface of recombinant-infected Sf9 cells indicates that E can travel to the cell surface independently of the occurrence of virus maturation processes. In flavivirus-infected cells anti-E fluorescence is also observed at the cell surface (Westaway and Goodman, 1987) but this may be explained by adsorption of released virus as electron microscopic studies of cells infected with YF, MVE, JE and DEN-2 (Ishak, *et al.*, 1988; Filshie and Rehacek, 1968; Hase *et al.*, 1987a; Hase *et al.*, 1987b) have each demonstrated virus on the surface of infected cells.

In most flavivirus-infected cells, virions appear to be assembled in the RER prior to release from the cell (Leary and Blair, 1980; Hase *et al.*, 1987a; Filshie and Rehacek, 1968; Ishak *et al.*, 1988). In view of this, E may be expected to be retained in some subcellular compartment for virus assembly. Thus transport of recombinant E to the surface of Sf9 cells may be due to the disruption of the assembly process. A surface localization has also been reported for expression of recombinant E of JE using baculovirus vectors, but not during the expression of DEN-4 structural proteins by vaccinia virus or baculoviruses (Zhao *et al.*, 1987; Bray *et al.*, 1989; Zhang *et al.*, 1988). Similarly DEN-2 E protein expressed using a vaccinia virus vector is not detected on the cell surface (Deubel *et al.*, 1988b). Possible explanations for these differences in distribution may include the level of expression of proteins, specific targeting signals present in the E proteins of different flaviviruses and the absence of other components of the assembly pathway. As the intracellular pathway of assembly of flaviviruses seems to favour retention of E in the membrane compartments where virion formation occurs, it will be of interest to investigate the factors mediating the transport

of E to cell surface in Sf9 cells and determine whether abberant transport occurred in Sf9 cells.

CHAPTER 6

CONCLUSION

This thesis has dealt with several aspects of the molecular biology of Murray Valley encephalitis virus. One aim of the work was to study functionally significant regions in the MVE E protein. Building an understanding of the structure and function of the E protein is of significance for a number of reasons. First, the early events of the infection process for MVE and other flaviviruses are not well-defined although it is clear that E has a role in cell binding and in fusion with the host cell membrane either at the cell surface or in the endosome. Second, the E protein is the main target for neutralizing antibodies which contribute to protection against infection in vertebrate hosts. Third, the signals which direct the E protein and its precursors through a morphogenetic pathway to assemble infectious virus particles have not been identified. One approach adopted was to define neutralization determinants in the envelope E protein since neutralization of infectivity may be mediated by interference with cell binding or fusion. In another approach, an expression system in *Spodoptera frugiperda* (Sf9) cells was set up to generate MVE E protein with a 'native' conformation with the aim of using the expressed protein and expression system to investigate the possible roles of E described above.

We attempted to map the epitopes defined by five neutralizing mAbs; the location of two of these was finally determined. A determinant of the E-8 epitope was mapped to residues 201-224 in E by deletion analysis of bacterial fusion proteins containing E fragments. The neutralizing anti-E-8 mAb, which cross-reacts with other viruses in the JE serogroup and cross-protects against JE and KUN, probably recognizes a linear neutralization determinant. Peptides which represent overlapping segments within 201-224 and site-directed mutagenesis targeted to the same region may be useful in defining the critical determinants of E-8.

A complementary approach of sequencing neutralization escape variants mapped the E-1c determinants to Phe 274, Ser 276 and Ser 277 in E. Together with previous findings that Ala 126 is part of the E-1c epitope, these results indicate that the E-1c

epitope is a 'discontinuous' epitope assembled from residues far apart in the linear amino acid sequence of E. Both E-1c and E-8 are in domain A, which may contain two other neutralization epitopes. On the primary sequence of E, E-8 is close to the A5 neutralization determinant of TBE. However both determinants of E-1c mapped here are distant from neutralization epitopes mapped for other flaviviruses; it is the only discontinuous epitope in flavivirus E proteins documented thus far. E-1c is defined by an mAb which neutralizes the infectivity of MVE in vitro and in vivo (Hawkes *et al.*, 1988). Investigation of the mechanism of neutralization may be of value in deciding whether E-1c participates in receptor binding. Alternatively, it is possible that anti-E-1c mAb may neutralize infectivity of MVE by preventing the acid-induced conformational change thought to precede or accompany fusion.

We have established that changes in the determinant of E-1c at Ser 277 and Ser 276 can modify the virulence of MVE for mice. The properties of these E-1c mutants point to a significant function for this epitope during infection and in the pathogenesis of MVE. Sequence analysis of additional escape variants and a more complete mapping of the residues involved in this epitope may therefore be of value in studies of the molecular pathogenesis of MVE.

Our study of the virulence of E-1c variants complements earlier reports that passaged variants of MVE and a neutralization escape variant of TBE with changes at Asp 390 and Tyr 384 respectively are attenuated in mouse virulence (Lobigs *et al.*, 1990; Heinz *et al.*, 1989). The role of Ser 277 and Asp 390 (which are in domains A and B respectively) in the function of E has yet to be defined. In more extensive studies of the significance of residue 277, it will be desirable to generate MVE mutants or MVE E proteins with specific changes at this residue or close by. The former can be achieved

through the use of an infectious cDNA clone for MVE which can be subjected to site-directed mutagenesis. Mutants generated would be useful in determining the effect on virulence of various changes at or near residue 277 and in pathogenesis studies where the tissue distribution and kinetics of spread of MVE mutants in mice can be compared.

In the work described, we have expressed the MVE structural proteins in insect cells using a baculovirus vector. A 54.5K protein was generated by proteolytic processing and Asn-linked glycosylation of the polyprotein precursor. From its reactivity with MVE E-specific mAbs and its size it was clear that a protein closely resembling E had been generated, although the N- and C-termini of the protein were not characterized. With a yield of 5 μg E per 10^6 cells, this expression system could be scaled up to produce 'native' MVE E protein for functional, structural and protection studies. Isolation and purification of the E protein may require detergent solubilization and use of immuno-affinity columns coupled with E-specific mAbs. Structural analysis and crystallization of E may thus be feasible. The question of whether E undergoes oligomerization or intermolecular association with prM during morphogenesis could be investigated by cross-linking studies and protease treatment.

The E protein from Sf9 cells, either purified or in crude lysates, could have other applications; these include the further definition of neutralization epitopes E-1d, E-5b, E-7 and E-8. Mutational analysis using the recombinant baculovirus construct could be employed to characterize these epitopes. We have generated a recombinant MVE transfer vector in this work which is amenable to mutation by restriction enzyme deletion or site-directed mutagenesis using M13 phage. Cotransfection of Sf9 cells with such mutant transfer vectors and baculovirus DNA would generate recombinant baculoviruses expressing mutant E proteins. Effective procedures for cotransfection and isolation of recombinant baculovirus have been set up in this work so that the strategy described above can be performed.

The capacity to generate mutant E proteins will also be of value in studying cell binding and fusion. In view of the significance of residues around Ser 277 and Asp 390, the effect of mutation (by deletion or amino acid change) of these residues on the cell binding activity and fusion activity of E are of interest. The candidate fusion region (residues 98-111) could also be mutated to examine its effect on fusion. Experimental procedures for studying fusion activity have not been established for flaviviruses but an indirect approach for determining changes in the conformation of E as judged by mAb reactivities and proteolytic cleavage patterns has been used for TBE and JE (Guirakhoo *et al.*, 1989). The expression of influenza virus haemagglutinin on the surface of monkey (COS) cells using the SV40 expression system has allowed a study of the acid-activated fusion process (Gething *et al.*, 1986; White *et al.*, 1982) by cell to cell fusion. Thus various approaches may be investigated with the baculovirus expressed E protein. We have shown using the recombinant MVE baculovirus construct that the MVE E protein, as cleaved from a larger precursor, is retained in the RER-Golgi region as in normal virus infection. A point of interest in future studies will be to determine what features of E determine retention in these subcellular compartments. Such studies could involve a variety of modifications to the E gene or to the adjacent proteins in the polyprotein.

In summary, the work reported in this thesis has contributed to our understanding of the structure of the flavivirus E protein and the regions of functional significance in E. We have developed a good expression system for the MVE E protein in insect Sf9 cells which will allow further investigation of the functional determinants of E and of its roles in pathogenesis and during infection.

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